The Dissertation Committee for Min Hui Lim Certifies that this is the approved version of the following dissertation:

Roles for extracellular nucleotides and apyrases in regulating primary root growth in Arabidopsis

Committee:

Stanley Roux, Supervisor

Mona Mehdy

Enamul Huq

Sibum Sung

Christine Hawkes

Roles for extracellular nucleotides and apyrases in regulating primary root growth in Arabidopsis

by

Min Hui Lim, B.S.; M.S

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Dedication

To Gangyee

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Roles for extracellular nucleotides and apyrases in regulating primary root growth in Arabidopsis

Min Hui Lim, Ph.D.

The University of Texas at Austin, 2014

Supervisor: Stanley Roux

When plant cells grow and when they are mechanically stimulated or wounded or attacked by pathogens, they release ATP into their extracellular matrix. This extracellular ATP (eATP) can induce cell signaling changes that alter cell growth and promote adaptive changes to biotic and abiotic stresses. Cells control the eATP concentration with ecto-phosphatases. Among the most important of these are the nucleoside triphosphate-diphosphohydrolases (NTPDases) called apyrases. There are seven apyrases in *Arabidopsis,* and at least two of these, APY1 and APY2, help control the eATP concentration. The expression of APY1/APY2 can be inhibited by RNAi, and this suppression leads to growth inhibition. Because prior work showed that increased levels of exogenous ATP can block auxin transport and inhibit the gravitropic response in primary roots of *Arabidopsis*, in this report I tested whether the expression of APY1/APY2 could influence auxin transport and gravitropism. When the expression of these apyrases was suppressed either genetically or chemically, indirect assays of auxin distribution in primary

roots showed that that polar auxin transport was interrupted and that, subsequently, the growth and gravitropic curvature of the roots were inhibited.

By microarray and qRT-PCR analyses, we assayed how the suppression of APY1/ APY2 by RNAi in R2-4A mutants changed the expression of genes linked to growth inhibition in seedlings. The most significant gene expression changes induced by apyrase suppression were in genes involved in stress responses, which included those regulating wall composition. These expression changes predicted specific chemical changes in the walls of mutant seedlings, and significant changes in reactive oxygen species (ROS) and wall lignification in roots were verified by direct analysis.

A group of class III wall peroxidases that are known to be stress response genes and involved in wall modification is up-regulated in the primary roots of R2-4A seedlings when the expression of APY1 and APY2 is suppressed. Those peroxidases use ROS substrates to produce cross-linking and lignification in cell walls. To verify their roles in root growth, a study of peroxidase knockout mutants was performed in the background of R2-4A mutants. When the expression of APY1/APY2 was suppressed in two of these mutants, *per54* and *per54/49,* their root growth was significantly greater than in R2-4A mutants expressing wild type peroxidases.

Taken together the results are consistent with the hypothesis that APY1/APY2 and eATP play important roles in the signaling steps that link polar auxin transport to growth, and stress-induced wall changes to growth inhibition.

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Chapter 1. Introduction

APY 1 and APY 2 control the growth of Arabidopsis

Apyrases are nucleoside triphosphate-diphosphohydrolases (NTPDases) that hydrolyze both nucleoside triphosphates and diphosphates, but not monophosphates. These are distinguishable from ATPases because of their low substrate specificity and insensitivity to classical inhibitors of ATPases (Komoszynski and Wojtczak, 1996). There are two main categories of apyrases, ecto-apyrases which are catalytically active in the extracellular matrix (ECM) (Plesner 1995) and endo-apyrases which are localized in the cell interior (Komoszynski and Wojtczak, 1996).

Seven apyrases have been identified in *Arabidopsis* (APY1–APY7), and Apyrase 1 (AtAPY1: At3g04080) and Apyrase 2 (AtAPY2: At5g18280) are most similar to the pea (Pisum sativum) ectoapyrase NTP9 (Steinebrunner et al., 2000). Those two apyrases have been localized in Golgi (Schiller et al., 2012; Chiu et al., 2012), but some fraction of them may also function as ecto-apyrases, since polyclonal antibodies raised to APY1/2 inhibit the growth of *Arabidopsis* pollen tubes and cotton fibers and raise the ATP concentration in culture medium of germinated *Arabidopsis* pollen (Wu et al., 2007) and cotton fibers growing out from cultured ovules (Clark et al., 2010a). They are 87% identical in protein sequence and have overlapping functions: a single knockout of APY1 or APY2 does not show any phenotype, but the double knockout *apy1apy2* is sterile because the pollen does not germinate in this mutant (Steinebrunner et al., 2003). To test the effects of suppression of APY1 and APY2 on growth, Wu et al. (2007) made transgenic plants in which the suppression of APY1 by RNAi could be induced in an *apy2* mutant. In these plants, the sense-intron-antisense mRNA of APY1 is transcribed when estradiol is applied, and this

decreases *APY1* transcript level to less than 30% compared to wild type levels in whole seedlings within 3.5d after adding the estradiol inducer, as estimated by RT-PCR (Lim et al., 2014). The major phenotypic changes occur in the root: swollen root tips, disappearance of elongation zone and inhibited polar auxin transport (Liu et al., 2012). Thus, genetic and phenotypic changes in the root due to apyrase suppression are highlighted in this report.

The other apyrases in the family of *Arabidopsis* apyrases, APY3 through APY7, have been cloned and characterized, but their physiological and biochemical functions are still not clear. Subcellular localization of APY3-APY7 was carried out using YFP-tagged transient expression in onion epidermal cells (Chui, Ph.D. dissertation 2013, University of Texas at Austin). The data showed that APY4, 5, and 7 expressions overlapped with the cis-Golgi marker, APY3-YFP displayed a small punctate pattern in the cytoplasm, and APY6 expression co-localized with an ER marker. The dissertation research of Jian Yang (Ph. D. dissertation 2011, University of Texas at Austin), revealed that there are no phenotypic changes in each single knock-out mutant of APY3, 4, and 5. He found, however, that APY6 and 7 are expressed in mature pollen and affect male fertility: each single knock-out of APY6 and 7 has abnormal extine structure, and the double knock-out of APY6 and 7 has collapsed pollen and defective extine, resulting in reduced seed set compared to WT (Yang et al., 2013). Even though they affect plant fecundity, APY6 and 7 do not affect plant growth.

The effects of extracellular ATP on plant

In addition to being an energy source inside cells, ATP is involved in cell signaling when it is released into extracellular matrix (ECM) both in animals (Corriden and Insel, 2010; Elliott et al., 2009; Noguchi et al., 2008; Schachter et al., 2008; Sluyter et al., 2004), and in plants (Chivasa

et al., 2005; Choi et al., 2014; Clark et al., 2010a and b; Clark et al., 2011; Liu et al., 2012; Roux and Steinebrunner, 2007; Song et al., 2006; Tang et al., 2003). Plant cells release ATP into their ECM when they are wounded (Song et al., 2006), mechanically stimulated (Jeter et al., 2004), and when they are actively secreting, such as during growth (Kim et al., 2006). Plants and animals control this eATP because it is such a potent regulator, and their cells respond to micromolar ATP changes (Demidchik et al., 2003; Song et al., 2006). The important roles of eATP in plant signaling have been shown both at the cellular level and at tissue levels: pollen tube growth (Reichler et al., 2009), stomatal aperture (Clark et al., 2011), cotton fiber growth (Clark et al., 2010a), root hair growth (Clark et al., 2010b), and gravitropic response and auxin transport in roots (Tang et al., 2003; Liu et al., 2012).

The effects of eATP are mediated by the signaling changes it induces. The application of 0.1 mM ATP to plants induces increased cytosolic Ca^{2+} levels (Demidchik et al. 2003, 2009, 2011; Jeter et al. 2004), increased reactive oxygen species (ROS) via the NADPH oxidases in *Arabidopsis* (Song et al. 2006; Clark et al., 2010; Clark et al., 2011), NO burst in tomato (*Solanum lycopersicum*) suspension cells (Foresi et al. 2007), in the pollen tubes (Reichler et al., 2009), root hairs (Clark et al., 2010), and guard cells (Clark et al., 2011) of *Arabidopsis thaliana*, and in two macroalgae, *Dasycladus vermicularis* and *Acetabularia acetabulum* (Torres et al., 2008). These eATP-mediated processes are initiated by purinergic receptors, including ligand-gated ion channels (P2X) and G protein-coupled receptors (P2Y) in animals. Until recently a plant receptor for eATP had not been identified, but finally one of them has been characterized by using an ATP-insensitive *Arabidopsis* mutant, *dorn1* (Does not Respond to Nucleotides 1) (Choi et al., 2014). They showed that DORN1 is a nucleotide-binding protein by an *in planta* ATP-binding assay using *Arabidopsis* protoplasts. They also compared gene expression changes between ATP application

and wounding response, and 60% of the gene expression changes induced by these two stimuli were matched. DORN1-overexpressed line expressed selected genes after wounding at a much higher level, while *dorn1* expressed those genes notably less. It suggests that eATP acts as a signal molecule when it is recognized by the DORN1 receptor kinase during plants' response to physical damage. However, although the function of DORN1 is linked to the rapid increase in $[Ca^{2+}]_{cyt}$ characteristically induced by eATP in plants, how the kinase activity of DORN1 promotes increased $[Ca^{2+}]_{cyt}$ remains to be elucidated. Also, *DORN1* knockout mutants do not have a growth phenotype, and if this means that DORN1 is unlikely to be the receptor for the documented growth and development changes induced by extracellular nucleotides, then additional eATP receptors may be discovered in the future.

Interestingly eATP plays an important role not only in cell growth and development, but also in programmed cell death (PCD), a process essential for growth and development changes in plants, such as seed and fruit production, and in responses to biotic and abiotic stresses to eliminate injured or infected cells. The PCD response, like other eATP effects, likely also result from a rapid increase in cytosolic Ca^{2+} levels, followed by ROS (hydrogen peroxide, H_2O_2) and NO accumulation. Then those secondary messengers increase MAP kinases that can regulate many downstream responses, including especially responses to biotic and abiotic stresses (Jeter et al., 2004; Song et al., 2006; Choi et al., 2014). The ROS can induce increased mitochondrial transmembrane potential (Demidchik et al., 2003; Sun et al., 2012). This can cause the release of cytochrome c from mitochondria into the cytosol and the activation of caspase-like proteases in cells, which are critical event to induce apoptotic-like PCD (Reape and McCabe 2008; Reape et al., 2008). The eATP-induced ROS can also modify ion channels, such as calcium influx channels, and potassium efflux channels. H_2O_2 treated root cells have activated K^+ efflux (Demidchik et al.,

2010), possibly because H_2O_2 with peroxidase produces hydroxyl radical (·OH) in cells (Chen and Schopfer, 1999), and hydroxyl radicals activate K^+ efflux conductance (Demidchik et al., 2003). Potassium is important in metabolic reactions and physiological processes since it is an essential nutrient. Losing potassium along with calcium influx in the cell stimulated by abiotic and biotic stress could induce plant PCD (Demidchik et al., 2010).

Role of ROS in primary root of Arabidopsis thaliana

Reactive oxygen species (ROS) are defined as "chemically reactive molecules containing oxygen" (Wikipedia), and common examples include superoxide, hydrogen peroxide, and hydroxyl radical. These ROS have signaling and regulatory roles in plants (Apel and Hirt, 2004; Demidchik et al., 2007; Dunand et al., 2007; Gapper et al., 2006; Mittler et al., 2004). They help control plant development by regulating cell growth (Foreman et al., 2003; Gapper and Dolan, 2006; Liszkay et al., 2004; Schopfer et al., 2002) and gravitropism (Joo et al., 2001). Additionally, ROS is produced during pathogen attack and abiotic stresses to regulate signaling and gene expression, and thus control developmentally regulated processes (Petrov and Van Breusegem, 2012; Shin and Schachtman, 2004; Shin et al., 2005; Trujilo et al., 2004).

The balance of different ROS is important for cell growth both in cells and at the tissue level. In animal cells, the control of cellular ROS (superoxide and hydrogen peroxide) levels by manganese superoxide dismutase (MnSOD) is critical for cell proliferation, i.e., an increase in superoxide steady-state levels by decreasing of MnSOD activity induces proliferation while the decrease in superoxide levels by an increase in MnSOD activity supports quiescent growth (Sarsour et al., 2008). In the *Arabidopsis* root, two ROS, hydrogen peroxide and superoxide, exhibit a characteristic distribution pattern: hydrogen peroxide mainly appears in the walls of cells

in the maturation zone, but superoxide accumulates in elongation and meristematic zones (Dunand et al., 2007). Hydrogen peroxide produced by peroxidases *in vivo* and *in vitro* promotes the formation and elongation of root hairs (Foreman et al., 2003), so the location of hydrogen peroxide in the root fits well with this role. Maintenance of O_2^- levels is important for normal cell growth (Dunand et al., 2007; Gapper and Dolan, 2006), and its presence in the elongation zone could allow it to participate in the loosening process (Chen et al., 1999). Moreover, Tsukagoshi et al. (2010) showed that increased superoxide level with decreased hydrogen peroxide level in root tips induces proliferation, but decreased levels of superoxide with increased hydrogen peroxide levels decreases the size of the meristem in root tips (Tsukagoshi et al., 2010).

Plant cell wall controls cell growth

The primary cell wall is a semi-rigid and polysaccharide-rich matrix which is synthesized during cell division, and it determines tissue and organ morphology. The secondary cell wall is a more rigid constuct added interior to the primary cell wall after cell growth ceases. Cell migration is not possible since plant cells are tightly glued to each other through their cell walls. Thus, plant form is determined by localized cell division and selective cell enlargement and by when those walls are synthesized, assembled and remodeled. The plant cell wall not only provides structural support and cell shape, but also helps regulate cell growth and responses to environmental and pathogen-induced stresses.

The initial cell wall (cell plate) is formed after nuclear division, then the cell plate fuses with the plasma membrane and becomes the dividing border between two new cells. Expanding cells are surrounded by a primary cell wall that stretches in response to turgor pressure as cells grow, but once cells reach their mature size, some of them, such as the conducting cells in xylem,

produce rigid secondary walls between the primary cell wall and the plasma membrane (Rose, 2003). The secondary cell wall contains lignin and is less flexible and less permeable to water than the primary cell wall, thus it makes walls more resistant to degradation and able to seal in water.

The polysaccharide and protein composition in the cell wall changes during cell division, elongation, and differentiation. Expansin is a cell-wall-loosening protein, and endoglucanases, xyloglucan endotransglucosylase/hydrolases (XTHs), and other enzymes alter wall structure to modulate expansin action (Cosgrove, 1999; Rose et al., 2004). Arabidopsis has 26 α-expansin genes and 5 β-expansins that are differentially regulated by environmental and hormonal signals (Lee et al., 2001). Those expansins are responsible for cell expansion, whereas cross-liking of extensins by peroxidases plays an important role in the restriction of cell growth (Brownleader et al., 2000). Even though the chemical structures of most wall components have been defined, the enzymes involved in their synthesis and remodelling are still largely unknown. Since the induction of apyrase suppression in R2-4A mutants results is growth inhibition, and cross-linking in the cell wall plays a critical role in cell wall stiffening, one of the most important wall enzymes for cross-linking, peroxidase, is a focus of this report.

Class III peroxidases

The peroxidases have numerous biosynthetic and degradative functions, and the superfamily of plant peroxidases is divided into three classes by their function and locations: Class I peroxidases are intracellular peroxidases and play an important role in the detoxification in the cytosol, chloroplasts, microbodies, and mitochondria (Córdoba-Pedregosa et al., 2003; De Gara, 2003; Shigeoka, 2002); Class II peroxidases are fungal enzymes involved in lignin degradation (Welinder and Gajhede, 1993); Class III peroxidases are the secretory plant peroxidases involved

in a broad range of processes, including cell wall stiffening (Iiyama et al., 1994), lignin and suberin deposition (Pomar et al., 2002; Quiroga et al., 2000), abiotic stress response (Almagro et al., 2008; Kim et al., 2010; Shin and Schachtman, 2004; Shin et al., 2005), biotic strees response (Daudi et al., 2012; Jin et al., 2011), auxin catabolism (Gazaryan et al., 1996) and biosynthesis of secondary metabolites (Awad et al., 2000; Sottomayor and Ros Barcelò, 2003). Their broad range of functions is due to their catalytical versatility and the great number of their isoforms (Passardi et al. 2005) .

In *Arabidopsis*, class III peroxidases have 73 isoenzymes (Tognolli et al., 2002). Their functions are still poorly understood because of their multiple levels of regulation, their broad substrate specificity, and their highly reactive products which later can participate in non-enzymatic reactions.

A major role proposed for wall peroxidases is to catalyse the dehydrogenative polymerization of monolignols, *p*-coumaryl, coniferyl, and sinapyl, into monomers of lignins (Lagrimini et al., 1987; Ostergaard et al., 2000). Lignins are phenolic biopolymers, and synthesized for mechanical support, defence and water transport in vascular plants. The co-localization of H_2O_2 and zones of lignification has been reported (Olson and Varner, 1993). Evidence for the involvement of peroxidases in lignification is abundant (Herrero et al., 2013; Quiroga et al., 2000; Sasaki et al., 2006). Among the specific class III peroxidases confirmed to be involved in lignification are AtPrx 4, 52, 49 and 72 . Moreover, ATP A2 (AtPrx 53, peroxidase 53) mRNA is detected mainly in roots, and is reportedly used for docking of the monolignols and the plant cell wall cross-linker component ferulic acid (Ostergaard et al., 2000).

A related function of wall peroxidases is to catalyze the cross-linking of iso-dityrosine and diferulate bonds, which, like lignification, contributes to wall stiffening. The reaction of purified

extensin with hydrogen peroxide and peroxidases can yield intermolecular isodityrosine linkages at pH 3.7-5 *in vitro*, and this helps to explain why tyrosine residues of extensin *in vivo* are oxidatively coupled to form isodityrosine bonds, since the pH of cell wall is lower than neutral (Fry, 1987; Held et al., 2004). This cross-linked amino acid, isodityrosine, is a key to extensin network insolublization, which makes cell wall stiffening for mechanical support and defense (Lamport et al., 2011).

Certain peroxidases expressed in roots play important roles in responses to wounding and pathogen attack, and in aspects of plant development. Transgenic tobacco plants that overexpress anionic peroxidases in their roots have diminished root mass, which contributes to plant wilting (Lagrimini, 1997). Both overexpression and knockout mutants of a member of the type III peroxidase family, RCI3, inhibit root growth (Kim et al., 2010), and two highly homologous Arabidopsis peroxidases, AtPrx33 and AtPrx34, are involved in the increase of cell elongation; i.e., loss-of-function mutants had roots shorter than wild type roots (Passardi et al., 2006). Also, those two peroxidase mutants have a decreased oxidative burst in response to pathogen attack (Daudi et al., 2012). Those studies show that the balance of expression of different peroxidases is critical for root growth and defence.

Note

The following two chapers contain data published in the journal *Plant Physiology*: some data in Chapter 2 are taken from Liu et al., 2012 and Lim et al., 2014; and some data in Chapter 3 are taken from Lim et al., 2014. Almost all the data presented in these Chapters were generated by me, and any data produced by others (e.g., co-authors) are explicitly noted.

Chapter 2. Role for Apyrases in maintenance of extracellular ATP and in Polar Auxin Transport in *Arabidopsis thaliana*

INTRODUCTION

In addition to being an energy source inside cells, ATP functions as a signaling agent. When it is released into extracellular matrix (ECM) it induces rapid increases in the concentration of cytosolic calcium that are transduced into downstream changes in cell physiology both in animals and in plants (Burnstock, 2007; Chivasa et al., 2005; Choi et al., 2014; Clark et al., 2010; Clark et al., 2011; Demidchik et al., 2011; Kim et al., 2006; Liu et al., 2012; Roux and Steinebrunner, 2007; Song et al., 2006; Tang et al., 2003). These downstream changes in plants include changes in pollen tube growth (Reichler et al., 2009), stomatal aperture (Clark et al., 2011), cotton fiber growth (Clark et al., 2010a), root hair growth (Clark et al., 2010b), and gravitropic response and auxin transport in roots (Tang et al., 2003). As several reports have revealed, the signaling changes induced by extracellular nucleotides can communicate with signaling changes induced by one or more of the hormones that regulate plant cell growth. Tang et al. (2003) showed that extracellular nucleotides affect auxin transport, resulting in the inhibition of gravitropic growth, and Clark et al. (2010a) showed that when the application of nucleotides to cotton ovules growing in culture altered the rate of cotton fiber growth, it also induced the production of ethylene, a hormone known to regulate the growth of cotton fibers.

It would be important for cells to control the concentration of these nucleotides because extracellular ATP (eATP) plays an important role not only in cell growth and development, but also in programmed cell death (PCD), a process essential for growth and development changes in

plants (Demidchik et al., 2010; Reape and McCabe 2008; Reape et al., 2008). In fact, plants and animals control this eATP using various ecto-phosphatases, such as apyrase, in response to micromolar ATP changes (Demidchik et al., 2003; Song et al., 2006). Apyrases are nucleoside triphosphate-diphosphohydrolases (NTPDases) that hydrolyze both nucleoside triphosphates and diphosphates, but not monophosphates. These are distinguishable from ATPases because of their low substrate specificity and insensitivity to classical inhibitors of ATPases (Komoszynski and Wojtczak, 1996). Seven apyrases have been identified in *Arabidopsis* (APY1–APY7), and two of these, APY1 and APY2, share 87% protein sequence identity but are less than 30% similar to the other five apyrases. Polyclonal antibodies raised to APY1/2 and mutant studies showed that these two apyrases partially complement each other's function and play central roles in growth control (Clark et al., 2010; Steinebrunner et al., 2000; Steinebrunner et al., 2003; Wu et al., 2007).

Although the suppression of APY1/APY2 or of apyrase activity has a dramatic effect on growth, overexpression of APY1 or APY2 has much less of an effect. Constitutive expression of APY1 induces a small but statistically significant increase in the growth of etiolated hypocotyls, while overexpressing APY2 has no effect on this growth (Wu et al., 2007). This is probably because the wild-type levels of apyrase expression are near optimal for growth (Roux and Steinebrunner, 2007). The single knockout of APY1 or APY2 does not show any phenotype, but the double knockout *apy1apy2* is sterile because the pollen does not germinate in this mutant (Steinebrunner et al., 2003). To test the effects of suppression of APY1 and APY2 on growth, Wu et al. (2007) made transgenic plants in which the suppression of APY1 by RNAi could be induced in an *apy2* mutant. This transgenic plant called R2-4A decreases APY1 transcript level to less than 30% compared to wild type levels in whole seedlings within 3.5d after adding the estradiol inducer

(Lim et al., 2014). Its pollen does germinate, permitting fertilization and subsequent normal development, however the adult plants of these mutants are dwarf (Wu et al., 2007).

This report revealed that the major phenotypic changes caused by apyrase suppression occur in the root: swollen root tips, disappearance of elongation zone, decreased cell numbers in meristematic zone and inhibited polar auxin transport. The inhibition of auxin transport in R2-4A was predicted since higher levels of eATP can inhibit auxin transport in roots (Tang et al., 2003). Consistent with this result and with the results of Tang et al. (2003), suppression of APY1/APY2 also blocks the asymmetric distribution of a GFP reporter encoded by a DR5:GFP construct in gravistimulated primary roots of *Arabidopsis* seedlings, and diminishes the extent of the elongation zone in these roots. These results are consistent with the novel conclusion that inhibition of auxin transport is a key step in the signaling pathway that links the inhibition of apyrase expression to growth inhibition.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Plant materials used in this study were *Arabidopsis thaliana* ecotypes Wassilewskija (Ws) and RNAi mutant line R2-4A. The RNAi construct, generation of the R2-4A line and growth conditions of the seedlings were as previously described (Wu et al., 2007). For estradiol treatment, estradiol was added into MS medium to reach a final concentration of 4 μM. Plates were placed upright in a culture chamber and grown at 23˚C under 24-h fluorescent light after 3 d vernalization at 4 ˚C.

Assay of [eATP] in Growth Media of Apyrase-Suppressed Seedlings

Seeds were planted on standard MS media with 1% sucrose and 1% agar. Twenty 3-d-old seedlings per well were transferred to liquid culture in six-well plates, which had 3 mL per well of MS media with 1% sucrose. Each plant line was grown in 4 µM estradiol prepared in DMSO to induce suppression of *APY1*, and control media with an equal volume of DMSO only. Each six-well plate contained six biological replicas of the same plant line and treatment, and the treatments were: wild type and R2-4A in DMSO only, wild type and R2-4A in 4 µM estradiol. One six-well plate was prepared for each of the days after planting that media samples were collected.

After transfer to liquid culture, seedlings were grown for the indicated number of days with constant shaking and constant light at 22-25°C. Two h before media collection the plates were removed from the shaker and kept still. Three individual samples of 50 µL of media were removed from each well and flash frozen in liquid nitrogen. Samples were stored in -40°C until ATP measurements were made. After media collection pictures were taken of several seedlings per treatment in order the measure root length and verify expected phenotypes (Lim et al., 2014).

The [ATP] in the growth media was determined using the ENLITEN ® ATP Assay System from Promega (Sunnyvale, CA). Reactions were carried out on 96-well plates and luminescence was detected using the Mithras LB940 microplate reader (Berthold Technologies). Media samples were thawed, placed on the plate quickly and kept on ice for ≤ 30 min before ENLITEN® reagent was added. In each reaction, 40 µL of ENLITEN® reagent was added to 10 µL of media sample. An ATP standard curve from 10^{-7} to 10^{-12} M was included on each plate. Lumen measurements were evaluated by the Dixon's Q test for outliers. In all cases at least 4 replica samples were included in the average.

Total RNA Isolation and Quantitative RT-PCR

6-d-old-seedlings were collected and frozen in liquid nitrogen. Plant tissues were ground to fine powder with a chilled mortar and pestle. Total RNA was isolated using Spectrum™ Plant Total RNA Kit (Sigma) according to the manufacturer's instruction. After treatment with DNase I (Invitrogen), total RNA (1µg) was reversely transcribed to cDNA using SuperScript II Reverse Transcriptase Kit (Invitrogen). cDNA (10ng) was amplified in real-time PCR reactions using SYBR Green PCR Master Mix (Applied Biosystems) with gene-specific primers (Table below). Real-time PCR was carried out in triplicate on each sample using an ABI 7900HT Fast Real-Time PCR System (Applied Biosystems). The relative expressions of genes were normalized to the level of a reference gene--protein phosphatase 2A (PP2A). The changes of transcripts were calculated using 2-ΔΔCT method (Shi and Chiang, 2005).

name	Primers	
PGP1	F	AGGCCGTCCTGACGCAGATCA
	R	TCCCCAACCTGTGTGTCGAAGC
PGP4	F	ATACGGCAAGACACCAAGAAGCCG
	R	CCTCACGATCCTCGAAAAGCAACAA
PGP19	F	GGAATCGCTTTCGCCGGAGGT
	R	TTGAGCAATTGCCTGCTCGGC
PIN1	F	CGGCGGCTATGAGATTTGTCGTT
	R	TTGCGGCAAAGCTGCCTGGA
PIN3	F	GCGTTGCAACCCAAATTAATCG
	R	CGTGATCGGAAGCGCTATAAGCAT
TIR1	F	CAGCGCCTCTGGGTGCTTGA
	R	TCAGCTCGCGTAGGTCCTTGC
ARF1	F	TGGTCCATCTGGTCCTGTTACTCCA
	R	ACGTAGCAGCTTTGGCAGGAGG
ARF2	F	CGGCGAACAAAAGATGATTACGGCG
	R	ACGGCGAAGAGAGCGACGAA
ARF3	F	ACCGCTGCTCTCTCGGCTCA
	R	CCAGCTTGCCTTGGGGTTGT

Confocal Microscopy

The liquid stock solution of propidium iodide (PI; SIGMA P4170) at 1 mg/ml was diluted for a staining solution of 5 µl/ml. The roots were stained in a dish of PI for 30 seconds to 3 minutes and then mounted on a microscope slide in water. 4',6-Diamidino-2-phenylindole (DAPI; SIGMA D9542) was prepared in water at 20 mg/ml. The roots were in darkness for few minutes with the DAPI staining solution (600 nM), then the DAPI stain was observed with UV excitation.

Both DR5:GFP R2-4A and DR5:GFP wild-type seedlings were transferred to plates containing 4 mM estradiol, 800 µM ATPγS, or apyrase inhibitor NGXT1913 on day 3. Fluorescence images were captured at day 6 by a Leica SP2 AOBS confocal microscope with the filters set at 488/509 nm for excitation/emission. For root gravitropic responses, seedlings were grown in light for 5 d and then rotated 90°. Images were obtained after 5 h of horizontal growth, and the confocal microscope noted above captured fluorescence images of the GFP signals. PIN2:GFP and AUX1:YFP wild-type seedlings were transferred to plates with and without 800 µM ATPγS to evaluate distribution of PIN2 and AUX1.

Apyrase Inhibitor Assays

Seeds (WS) were sterilized as described by Tang et al. (2003), kept at 4°C for at least 3 d, and then sown on MS medium with or without 15.75mg of the apyrase inhibitor, NGXT1913 (Windsor et al., 2002) or NGXT191 (Windsor et al., 2003), which was dissolved in dimethyl sulfoxide. The control plates contained the same final concentration of dimethyl sulfoxide (0.01%) as the treatment plates. Seedlings were grown in darkness for 3 d on vertically oriented plates, then the plates were moved into continuous white light, reoriented 90°, and the seedlings were grown for an additional 18 h. The growth temperature throughout was 23°C. Seedlings were imaged

before being reoriented and 18 h later, and ImageJ was used to measure changes in the angle of curvature and overall growth between these two time points.

RESULTS

Apyrase Suppression Results in Increased [eATP] in Growth Media

In Ws wild-type and induced R2-4A samples the [ATP] began to differ one day after transfer to liquid growth conditions with estradiol (Fig. 2.1 A). In growth media of R2-4A plants the [ATP] was higher than that of wild-type plants at all points tested, and the difference became greater coincident with decreased *APY1* expression (Fig. 2.1 B). This difference steadily increased up to 2-fold through 4 d, in parallel with decreased *APY1* expression. In contrast the [eATP] in the growth media of Ws wild type and un-induced R2-4A plants did not differ through 4 d of growth (data not shown). The [ATP] measured in the media is a very large dilution of the [ATP] likely to be present immediately to the outside of the plasma membrane, probably more than a thousand-fold (Song et al., 2006). The R2-4A plants grown in liquid culture had the expected root length and root tip phenotypes same as the root grown on agar plate. The experiment was performed three times, with six biological replicas of each sample included each time. Results were similar between all experiments; data from one experiment is shown in Fig. 2.1 A.

Both Cell Elongation and Mitosis Are Inhibited When Apyrase Expression Is Suppressed in Primary Roots

After R2-4A plants were treated with estradiol, there was a reduction in the overall length of their primary roots (Wu et al., 2007) due to a combination of factors. In R2-4A plants, the length

of the elongation zone disappeared. This can be determined since root hairs grow only in the differentiation zone, and it started showing near the root tip in R2-4A plants (Fig. 2.2 A). With DAPI staining, which stains DNA, plants with reduced apyrase expression displayed diminished cell number in the meristematic zone of the primary root (Fig. 2.2 B). Reduction of cell numbers in meristematic zone happened gradually, and slower than the disappearance of the elongation zone. The data reveal that after apyrase suppression differentiation is induced faster and mitosis is inhibited continuously in the primary root.

The Root Gravitropic Response Is Altered by Genetic Suppression of Apyrase Expression and Chemical Inhibition of Apyrase Activity

We tested whether *APY* suppression altered the root gravitropic response. In R2-4A roots that were not treated with estradiol, there was asymmetry of GFP fluorescence, similar to that observed in DR5:GFP WS wild-type roots (data not shown). In contrast to DR5:GFP seedlings that have normal APY1 and/or APY2 expression, there was no asymmetry of GFP fluorescence in RNAi-suppressed roots of DR5:GFP-expressing seedlings after gravistimulation (Fig. 2.3, A and B; performed by Jian Wu). A similar result was observed in wild-type DR5:GFP seedlings when they were treated with 800 mM ATPγS or with the apyrase inhibitor NGXT1913 (i.e. in these seedlings, there also was no asymmetry of GFP signal after gravistimulation; Fig. 2.3, C–F). These results suggest that apyrases and extracellular nucleotides may play a role during root gravitropism, so we gravistimulated both RNAi-suppressed plants and WS wild-type plants treated with a chemical inhibitor or apyrase inhibitor, NGXT1913 (Windsor et al., 2002). The growth of estradiol-induced R2-4A roots is significantly decreased in comparison with WS wild-type roots (Wu et al., 2007), and this growth inhibition would be expected to also inhibit gravitropic growth,

which is what we observed (Fig. 2.4). Note, however, that the gravitropic response is decreased even when an apyrase inhibitor does not affect growth. Treatment with the apyrase inhibitor NGXT1913 (Windsor et al., 2002) decreased the gravitropic angle of WS roots when they were reoriented relative to the gravity vector, but its effect on growth was not significant (Fig. 2.5). A similar result (data not shown) was obtained with another apyrase inhibitor, NGXT191. These results indicate that apyrase activity also contributes to the lateral transport of auxin that is needed for the gravitropic response. Note also that the DR5:GFP signal is strong in the root tip, indicating that when *APY* suppression results in reduced auxin transport shootward this is not due to inhibition of rootward auxin transport (Fig. 2.3; Liu et al., 2012).

Then I tested if there is any accumulated effect when NGXT191 and ATPγS are treated together in seedlings. The apyrase inhibitor has a greater inhibitory effect than ATPγS on the gravity response (Fig. 2.6 A and B), but neither treatment induced morphological changes near the root tip like those observed in R2-4A roots (Fig. 2.6 C and D).

Growth-Inhibiting Levels of Applied Nucleotides Do Not Alter the Localization of PIN1, PIN2, AUX1, or ABCB19 Transporters, and the Expression of PGP1, PGP4, PGP19 (ABCB19), PIN1, PIN3, TIR1, ARF1-3 is Not Changed by Genetic Suppression of Apyrase

Dose-response assays of the effect of applied ATPγS on the growth of etiolated hypocotyls and light-grown roots revealed that concentrations between 500 and 800 mM were needed to have a significant inhibitory effect (Roux et al., 2006; Tang and Roux, unpublished data). However, 800 mM ATPγS treatment did not alter the distribution of either PIN1 or ABCB19, which participate in acropetal IAA transport (Liu et al., 2007), or PIN2 and AUX1, which participate in basipetal IAA transport, in light grown roots (Fig. 2.7). Additionally, ATPγS treatment significantly

inhibited the elongation growth of primary roots in *pin2* and *aux1* mutants just as it did in wild-type seedlings (data not shown).

Additionally, I used quantitative reverse transcription-PCR to examine potential expression changes for PGP1, PGP4, PGP19 (ABCB19), PIN1, PIN3, TIR1, ARF1-3. There were no significant differences in the message levels of any of these auxin related genes (Table 2.1).

DISCUSSION

In etiolated hypocotyls, pollen tubes, cotton fibers, and root hairs, low concentrations of applied poorly hydrolyzable nucleotides (ATPγS or ADPβS) promote growth, and higher concentrations inhibit growth, so there appears to be an optimal [eATP] for growth (Clark et al., 2010a, 2010b; Reichler et al., 2009; Roux and Steinebrunner, 2007; Wolf et al., 2007; Wu et al., 2007). These dose-response results are qualitatively similar to the bimodal dose-response curves obtained using auxin (Mulkey et al., 1982) or ethylene (Pierik et al., 2006). APY1 and APY2 are highly expressed in rapidly growing tissue, and may function in part as ecto-apyrases to regulate eATP concentration in ECM, i.e. to maintain optimal eATP levels for normal plant growth (Wolf et al., 2007; Wu et al., 2007).

To clarify the role of APY1/APY2 as ecto-apyrases, we measured eATP levels released by WT and R2-4A seedlings while they are growing in liquid media. The [ATP] in the growth media of estradiol-induced R2-4A seedlings was higher than WT at all points tested, and this difference steadily increased up to 2-fold through 4 d, in parallel with decreased *APY1* expression. This result is consistent with the conclusion that APY1/APY2 play a role in regulating [eATP], and that the

increased [eATP] that results from apyrase suppression can trigger some cell signaling, for example the signal pathway related to growth inhibition in R2-4A plants as described below.

There is an overall growth inhibition in hypocotyls and roots when the expression of apyrase is suppressed in R2-4A plants (Wu et al., 2007). I focused on the primary root more closely since this tissue was the one that showed the most rapid and most obvious phenotypic changes in seedlings grown in both light and dark. As observed by the light microscope, the root showed abnormal root morphology: disappearance of elongation zone, spatial abnormal root hair growth, and bulging shape of epidermal cells. The confocal microscope revealed other root changes: variably-shaped cortex and epidermal cells near root tip, and reduced cell numbers in meristematic zone. Specifically, measurement of cell lengths in these zones showed that R2-4A plants had much less uniform cell sizes than WS wild-type plants, a difference that was quantified by showing that 20% more of the cells in R2-4A plants were smaller than one-half the mean diameter compared with WS wild-type plants (Liu et al., 2012). These results indicate that the reduction in length of R2-4A roots was due to both fewer cells in the mitotic zone and elongation zone and less expansion in the elongation zone.

Given the complex interaction of signaling pathways, the steps that lead from eATP to growth control are likely to intersect at some point with transduction steps induced by hormones. The study already showed that applied nucleotides block the auxin transport in maize and *Arabidopsis* root (Tang et al., 2003). We also tested asymmetric redistribution of auxin after gravistimulation by using wild-type DR5:GFP seedlings treated with 800 mM ATPγS or with the apyrase inhibitor, NGXT1913. The roots of treated seedlings showed less asymmetric auxin redistribution and less curvature in response to gravistimulation than the root of seedlings without treatments. Since we confirmed increased [eATP] in growth media of R2-4A, we hypothesize that

apyrase suppression could affect auxin transport, just as increased [eATP] does (Tang et al., 2003). We demonstrate that both in hypocotyls and roots, reduction in apyrase expression using RNAi leads to reductions in basipetal auxin transport in these tissues, the same tissues in which growth is inhibited by apyrase suppression (Liu et al, 2012). Consistently, the suppression of APY by RNAi in DR5:GFP-expressing seedlings showed no asymmetry of GFP fluorescence and no gravity growth in response to gravity in the primary root after gravistimulation. Taken together, the data suggest that the growth suppression by APY mRNA interference is at least partially due to auxin transport inhibition.

The inhibition of auxin transport could have occurred because of the expression changes of genes involved in auxin transport and/or auxin response. AUXIN RESISTANT1/LIKE AUXIN1 (AUX1/LAX) influx carriers, PIN-FORMED (PIN) efflux facilitators, and ATP-binding cassette subfamily B (ABCB, PGP) transporters are well-known protein families involved in polar auxin transport (Peer et al., 2011). Additionally, auxin receptor (TIR1) (Dharmasiri et al., 2005) and auxin response factors (ARFs) are important for auxin response. To test this hypothesis, I used quantitative reverse transcription-PCR to examine potential expression changes of these genes. Neither aerial tissue nor root tissue showed any significant differences in the message levels of those genes in any day tested after apyrase suppression. However, the data do not rule out the possibility that apyrase suppression could alter the posttranscriptional activity of auxin transporters. In fact, the level of nitric oxide (NO) is increased by applying nucleotides in plant cells (Foresi et al., 2007; Torres et al., 2008; Wu and Wu, 2008; Reichler et al., 2009; Clark et al., 2010b), thus induced [eATP] by apyrase suppression could regulate auxin signaling by increased NO level. Terrile et al. (2011) found that the nitrosylation of TIR1 regulates the degradation of AUX/IAA proteins. Moreover, another study showed that PIN1-dependent auxin transport is

inhibited by high levels of NO in Arabidopsis root (Fernández-Marcos et al., 2011). This would provide a mechanistic basis for understanding the link between the suppression of APY1/APY2 and the inhibition of polar auxin transport.

Surprisingly, the treatment of eATP and/or apyrase inhibitors in WT roots does not mimic the root morphological changes in estradiol-treated R2-4A roots (Fig. 2.6 C and D). Thus, even though suppressing APY1 and APY2 expression increases eATP levels and disrupts basipetal auxin transport, it seems probable that the R2-4A root phenotype may be due to the effects of suppressing apyrase expression that are not directly related to an increase in the eATP concentration. Gene expression and protein analysis should be performed to elaborate the mechanism of growth inhibition by apyrase suppression.

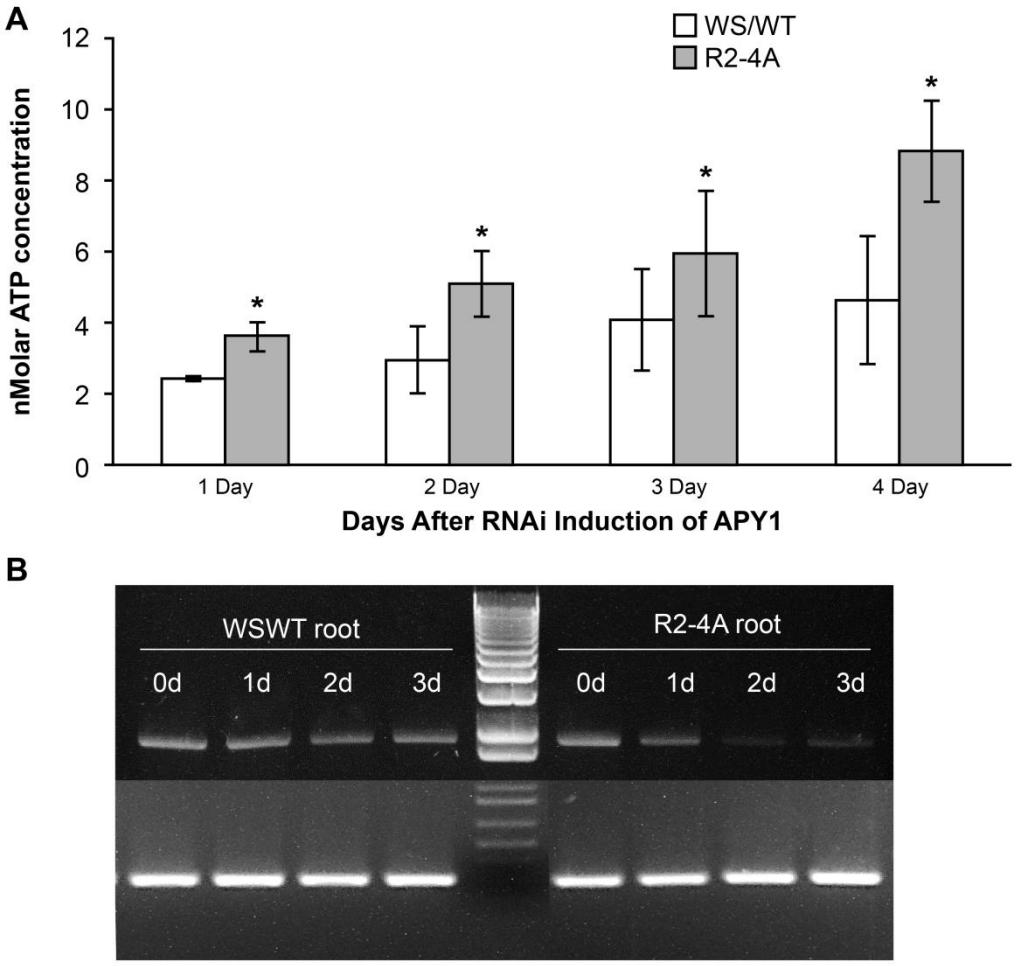

Figure 2.1. Media [eATP] Rises with Increased Suppression of Apyrase Expression in R2-4A Seedlings. (A) Time course of increase of [ATP] in seedling growth media, based on luciferase assay luminescence in comparison to ATP standard curve. Error bars represent standard deviation, and asterisks indicate statistical significance based on p value >0.05 in a Student's t-test (B) Time course of loss of APY1 transcripts during continuous treatment of R2-4A mutants with estradiol inducer of RNAi construct (upper lane) and actin (lower lane).

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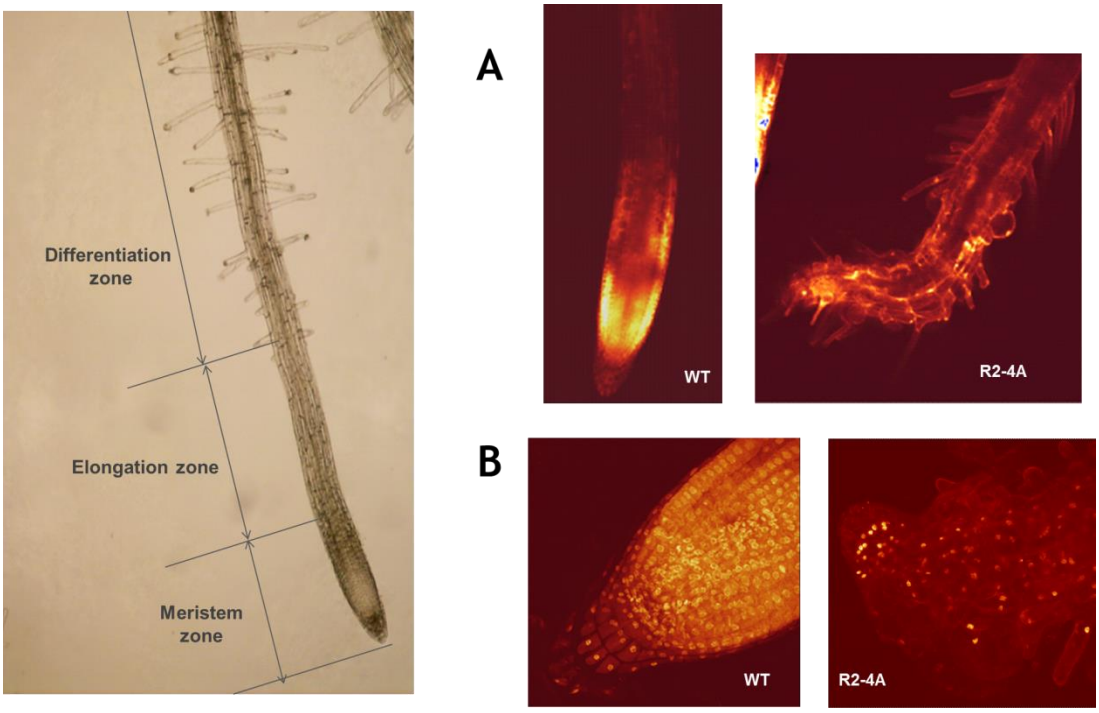

Figure 2.2. Morphological changes in primary root of seedlings by apyrase suppression. A, Elongation zone virtually disappears in R2-4A, so that root hairs begin to appear near the root tip. Confocal microscopy with propidium iodide (which stains the outlines of live cells) staining of 8-d-old primary root. B, Meristematic zone of R2-4A root has a dramatically reduced number of cells. Confocal microscopy with DAPI staining of 13d-old primary roots

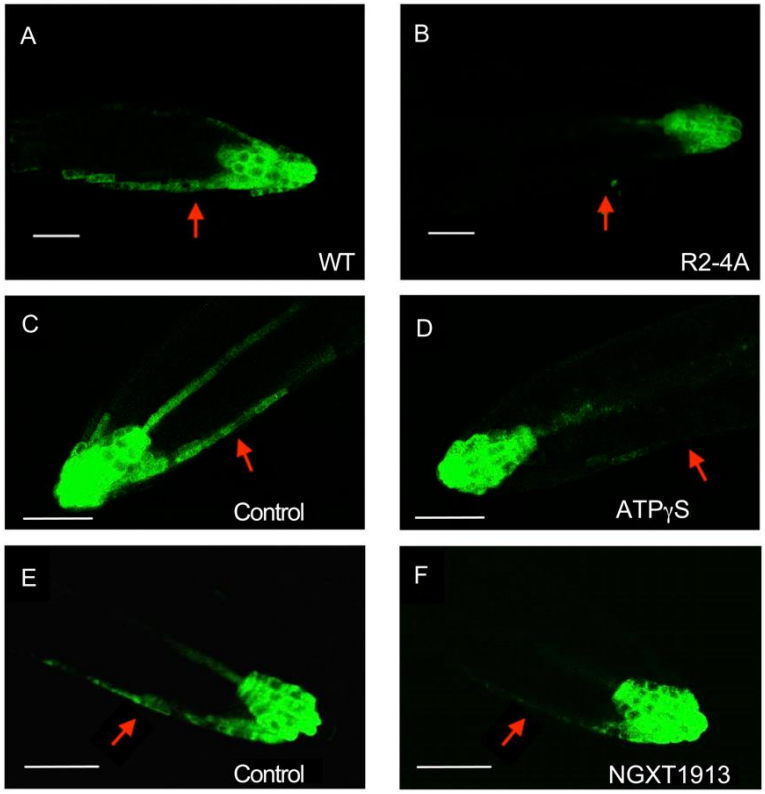

Figure 2.3. A and B, Fluorescent GFP signals in horizontally positioned primary roots of wild-type (WT; A) and R2-4A (B) plants expressing DR5: GFP. Both wild-type and R2-4A plants were treated with estradiol, which induced the suppression of apyrase expression by RNAi in the R2-4A mutant. C to F, GFP signals in horizontally positioned primary roots of wild-type plants expressing DR5:GFP before and after treatment with 800 mM ATPγS (C and D) or 15.75 mg of NGXT1913 (E and F). Arrows indicates the lower flank of the primary root, where the GFP signal is evident in the epidermal cells of the wild-type roots but not in the R2-4A roots and not in treated roots. These results are representative of 10 or more biological repeats. Signals were assayed 5 h after roots moved to the horizontal position. Bars = 50 mm.

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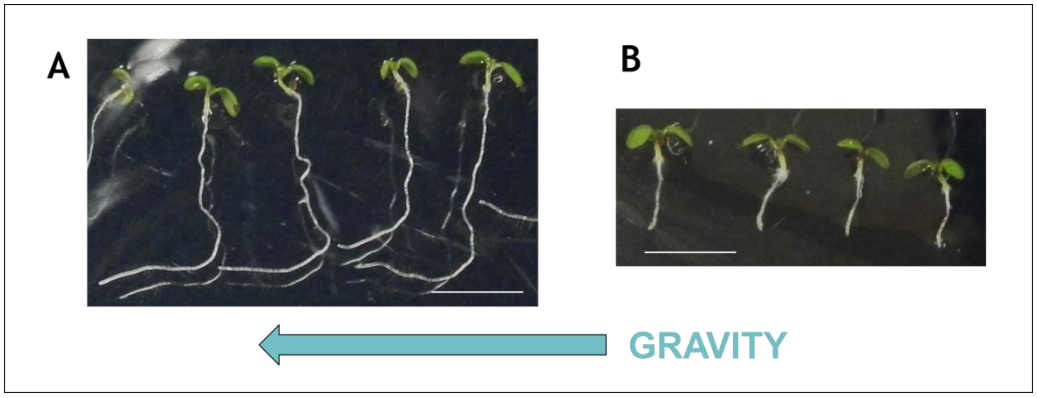

Figure 2.4. Gravity response of the primary root of WT (A) and R2-4A (B) on MS media with estradiol. 5d-old seedlings were moved to the horizontal position, then were grown 2d more. (scale bars = 5mm)

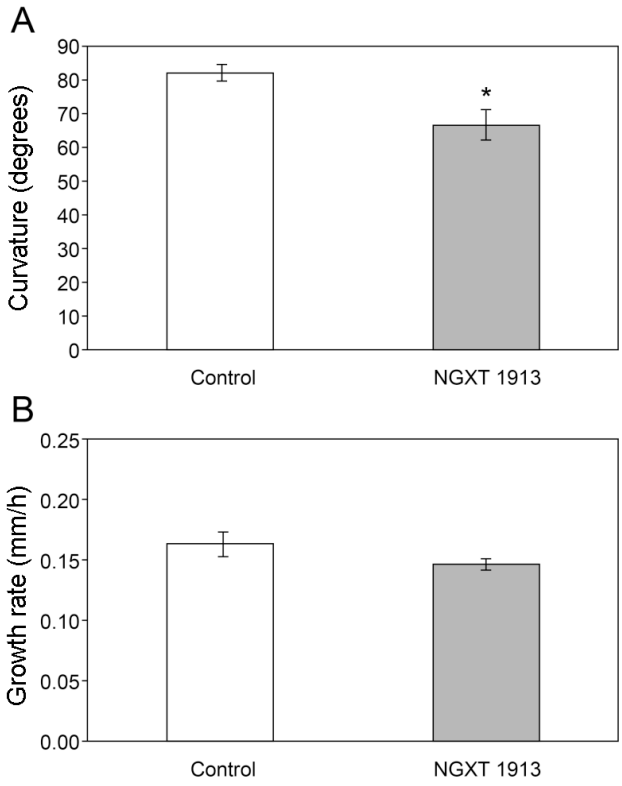

Figure 2.5. Chemical inhibition of apyrase activity blocks gravitropic growth of primary roots of Arabidopsis. A, Treatment of 3-d-old seedlings with 15.75 μg/mL NGXT 1913 inhibits root gravitropic curvature. B, Treatment of 3-d-old seedlings with 15.75 μg/mL NGXT 1913 reduces the root growth rate, but this effect is not statistically significant ($p = 0.332$). These data are the average of two independent experiments. Standard error values are marked by vertical bars. Significant differences between samples are indicated by asterisks (Student's t test; * $P < 0.001$; $n \geq 20$).

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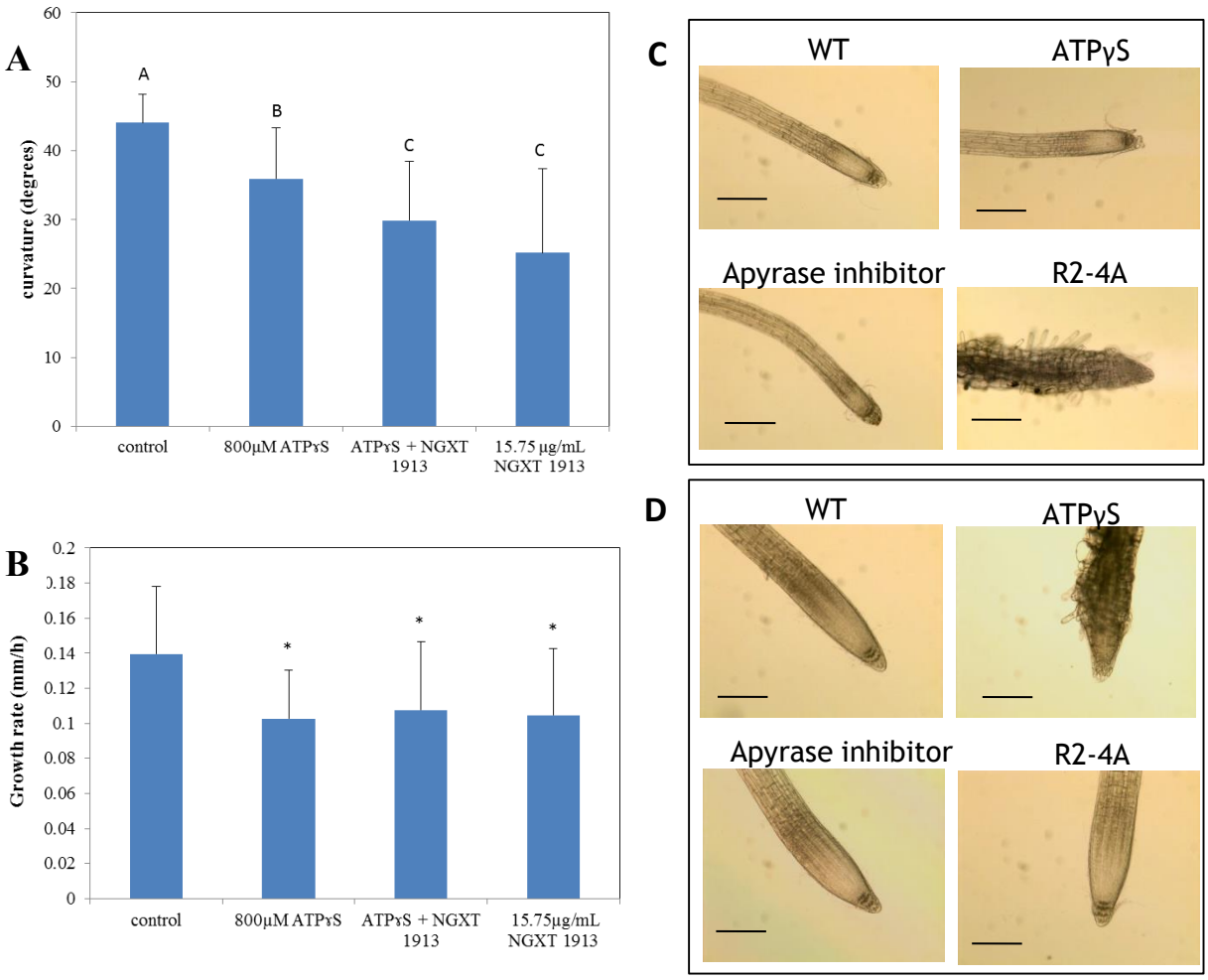

Figure 2.6. Comparison of chemical inhibition effects of apyrase inhibitor and ATPγS on primary roots of Arabidopsis. A, Treatment of 3-d-old seedlings with 15.75 µg/mL NGXT 1913 and/or 800µM ATPγS inhibits root gravitropic curvature in 3h. B, Treatment of 3-d-old seedlings with 15.75 µg/mL NGXT 1913 and/or 800µM ATPγS reduces the root growth rate. Standard deviation values are marked by vertical bars. Significant differences between samples are indicated by letters (P value < 0.01; n ≥20) and asterisks (* P < 0.001; n ≥20). C and D, WS wild-type roots treated with 15.76 µ M apyrase inhibitor or 800 µM ATPγS were no any morphological differences unlike R2-4A (C: 6d-old dark grown root; D: 6d-old light grown root; scale bars = 1mm)

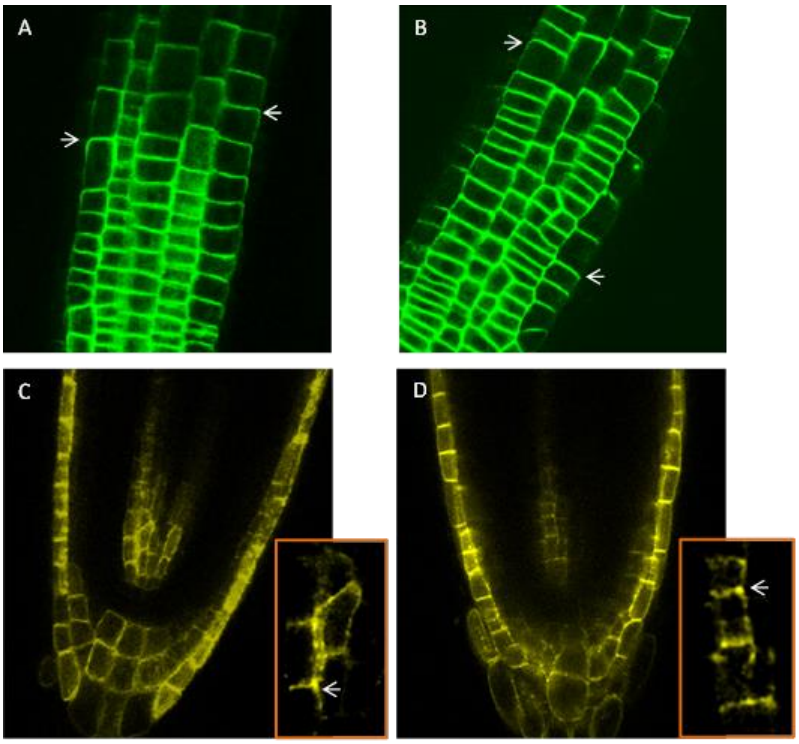

Figure 2.7. Distribution of neither PIN2:GFP nor AUX1:YFP is altered in Arabidopsis primary roots by treatment with 800 μM ATPγS. Confocal images of primary roots of 5-d-old seedlings expressing PIN2:GFP before (A) and after treatment with 800 μM ATPγS (B). Confocal images of primary roots of 5-d-old seedlings expressing AUX1:YFP before (C) and after treatment with 800 μM ATPγS (D). * Arrows indicate the polarity of PIN2:GFP and AUX1:YFP, respectively.

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A. qRT-PCR with aerial tissues

	PGP1	PGP4	PGP19	PIN1	PIN3	TIR1	ARF1	ARF2	ARF3
0d	0.898	1.054	0.870	0.829	1.123	0.740	0.992	0.994	0.893
1d	0.925	0.759	1.153	0.766	1.121	0.906	0.843	0.863	0.869
2d	1.097	0.865	1.051	1.006	1.206	1.175	1.228	0.887	0.926
3d	0.953	1.183	0.981	1.184	0.880	1.147	1.211	1.220	0.908

B. qRT-PCR with primary root tissues

	PGP1	PGP4	PGP19	PIN1	PIN3	TIR1	ARF1	ARF2	ARF3
0d	0.720	1.105	0.912	0.954	1.176	0.937	0.806	1.073	1.083
1d	0.629	0.994	0.950	1.045	0.989	0.895	1.232	0.922	0.961
2d	0.684	0.946	0.923	0.989	0.795	1.062	0.859	0.889	0.808
3d	1.156	1.040	1.012	1.381	1.024	1.259	1.296	1.594	1.382

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Table 2.1. No significant differences in gene expression changes of auxin transporters/carriers in R2-4A. qRT-PCR data checked the genes in aerial (A) and root (B) tissues separately. Treatment of estradiol (0d, 1d, 2d, and 3d) begins with 3-d-old seedlings of WT and R2-4A in the light. *Numbers represent average fold changes of three biological repeats in R2-4A compared to WT.

Chapter 3. Wall peroxidases associated with stress responses are upregulated by apyrase suppression in primary roots of *Arabidopsis thaliana*

INTRODUCTION

Plant cells release ATP into their extracellular matrix (ECM) or into the growth medium when they are wounded (Song et al., 2006), touched or mechanically-stimulated (Jeter et al., 2004; Weerasinghe et al., 2009), and as they grow (Kim et al., 2006; Wu et al., 2007). Dose-response assays indicate that extracellular ATP (eATP) can modulate the rate of cell growth, with low concentrations promoting growth and higher concentrations inhibiting it (Roux and Steinebrunner, 2007; Clark and Roux, 2009). It has been shown that ecto-NTPDases are key regulators of the concentration of extracellular ATP ([eATP]) in animals (Knowles, 2011). Those data indicate that eATP is involved in cell signaling, so controlling its concentration with enzymes is important for cell regulation.

In plants, two apyrases in *Arabidopsis*, APY1 and APY2, partially complement each other's functions. One of their functions is to serve as an ecto-apyrase to hydrolyze eATP, and this is one of the ways they regulate growth (Wu et al., 2007; Lim et al., 2014). Polyclonal antibodies raised to APY1/2 raise the [eATP] and inhibit the growth of pollen tubes in the culture medium of germinated pollen (Wu et al., 2007), and the inducible suppression of *APY1* by RNAi in the background of an *apy2* knockout in R2-4A mutants, also increases [ATP] in liquid growth media of seedlings (Lim et al., 2014). Their Golgi localization (Chiu et al., 2012; Schiller et al., 2012) raises the possibility that APY1 and APY2 could control the luminal [ATP] in secretory vesicles and thus indirectly control the [eATP], since one source of eATP is secretory vesicles that

have a high [ATP] in their lumen (Rudnick, 2008; Geigenberger et al., 2010). Finally, the treatment of 800 μM or higher concentration of ATP or ATPγS on WT plants mimics the inhibitory effects on growth of apyrase suppression (Tang et al., 2003). Those studies are consistent with hypothesis that a high [ATP] inhibits cell growth, and APY1 and APY2 can play an important role to limit the [ATP] in Golgi or both in Golgi and on the plasma membrane.

While a common strategy in response to physical stress in animals is 'run away' from a stressful situation, plants, being sessile organisms, cannot do this. They have developed their own stress response mechanism through cellular and molecular processes. Although an initial molecular process may be unique in distinct stresses, those different stresses could cause similar morphogenic response in plants, the so-called stress-induced morphogenic responses (SIMRs) (Potters et al., 2007 and 2009; Zolla et al., 2010). Many studies show that the SIMRs in roots involve common molecular processes, such as altered ROS production and hormones (Apel and Hirt 2004; Pierik et al., 2006; Romera and Alcantara, 1994; Schmidt and Bartels, 1996; Schmidt et al., 2000; Zolla et al., 2010). Both wounding and pathogen attacks would allow cytoplasmic ATP to leak into the ECM of plant cells, and this increased [eATP] would induce increased cytosolic Ca^{2+} levels, and increased reactive oxygen species (ROS).

Abiotic stresses such as salt, UV, drought, heavy metals, and air pollutants also induce the overproduction of ROS in plants. ROS produced during pathogen attack and abiotic stresses regulate signaling and gene expression, and thus control developmentally regulated processes (Petrov and Van Breusegem, 2012; Shin and Schachtman, 2004; Shin et al., 2005; Trujilo et al., 2004). The SIMRs in roots involve common physiological processes such as altered ROS production, hormone transport changes, inhibition of elongation and consequent formation of root hairs near the apex, and blocked cell division in the primary meristem (Potters et al., 2007). All

of these changes have been documented in the roots of estradiol-induced R2-4A seedlings (Wu et al. 2007; Liu et al., 2012). This implies that increased [eATP] by the suppression of APY1 and APY2 could induce gene expression and growth inhibitory changes similar to those induced by biotic and abiotic stresses. Here we test this prediction.

Abiotic and biotic stresses in plants also lead to changed lignin content and composition in plant cell walls (Moura et al., 2010). Lignin is the second most abundant biopolymer after cellulose, and it is important for structural integrity in secondary cell walls for mechanical support, protecting plants against pathogens, and helping to seal xylem for water transport in vascular plants. Monolignols such as p-coumaryl, coniferyl, and sinapyl undergo dehydrodimerization or polymerization with wall peroxidases and hydrogen peroxide to form monomers of lignins (Herrero et al., 2013; Lagrimini et al., 1987; Olson and Varner, 1993; Ostergaard et al., 2000; Quiroga et al., 2000; Sasaki et al., 2006; Zhang et al., 2012). Wall peroxidases are also important enzymes for functions other than lignification, for they are involved in a broad range of processes, including cell wall stiffening (Iiyama et al., 1994), lignin and suberin deposition (Pomar et al., 2002; Quiroga et al., 2000), abiotic stress responses (Almagro et al., 2008; Kim et al., 2010; Shin et al., 2004; Shin et al., 2005), biotic stress responses (Daudi et al., 2012; Jin et al., 2011), auxin catabolism (Gazaryan et al., 1996), and biosynthesis of secondary metabolites (Awad et al., 2000; Sottomayor and Ros Barcelò, 2003). Here the role of wall peroxidases in lignin formation and in transducing the suppression of apyrase into the inhibition of primary root growth in R2-4A mutants is tested and highlighted.

MATERIALS AND METHODS

Plant materials and growth conditions

Plant materials used in microarrays were *Arabidopsis thaliana* ecotypes Wassilewskija (WS) and the RNAi mutant line R2-4A, which was generated previously (Wu et. al., 2007). Seeds were surface sterilized and planted on Murashige and Skoog (1962) medium (4.3 g/L Murashige and Skoog salts (Sigma), 0.5% MES, 1% sucrose, and 1% agar, pH 5.7). For estradiol treatment, estradiol was added into MS medium to reach a final concentration of 4μM. Plates were placed upright in a culture chamber and grown at 23° C under 24-h fluorescent light after 3 days vernalization at 4°C. Light-grown seedlings were collected at day six. For dark-grown seedlings, plates were wrapped with aluminum foil and placed in the same growth chamber. Dark-grown seedlings were harvested at three and half days.

Qualitative RT-PCR

Six-day-old light grown and 3.5-day-old dark grown seedlings were used. Total RNA was extracted using Spectrum™ Plant Total RNA Kit (Sigma) according to the manufacturer's instruction. After treated with DNase I (Invitrogen), total RNA (1 μg) was reversely transcribed to cDNA using SuperScript II Reverse Transcriptase Kit (Invitrogen). cDNA was amplified in real-time PCR reactions using SYBR Green PCR Master Mix (Applied Biosystems) with gene-specific primers (see supplement material). Real-time PCR was carried out in triplicate on each sample using an ABI 7900HT Fast Real-Time PCR System (Applied Biosystems). The relative expressions of genes were normalized to the level of a reference gene-protein phosphatase 2A (PP2A). The changes of transcripts were calculated using $2^{-\Delta\Delta CT}$ method (Shi and Chiang, 2005).

Cell wall isolation and wall protein extraction

The cell wall was isolated from the primary roots of seedlings according to Feiz et al. (2006), with some modifications. Roots were ground with liquid nitrogen and incubated with extraction buffer 1 (5 mM sodium acetate pH 4, 0.4 M sucrose and 1X protease inhibitor cocktail) on ice for 30 min. The mixture was centrifuged at 1000*g* for 15 min at 4 ºC, and the pellet was resuspended in extraction buffer 2 (5 mM sodium acetate pH 4, 0.6 M sucrose and 1X protease inhibitor cocktail). The mixture was vortexed for 15 sec and incubated on ice for 2 min, then a pellet was collected by centrifugation. The step with extraction buffer 2 was repeated one more time, then this step was repeated with extraction buffer 3 (5 mM sodium acetate pH 4, 1 M sucrose and 1X protease inhibitor cocktail) instead of extraction buffer 2. Final pellet was washed with 5 mM sodium acetate pH 4 through 20 µm nylon membrane and collected for wall protein extraction.

To extract the ionically bound protein (IBP) fraction, the pellet was suspended in a high-salt extraction buffer (1 M NaCl, 50 mM sodium acetate, and 1X protease inhibitor cocktail) and incubated for 30 min on ice, and centrifuged at 5000 *g* for 15 min at 4 ºC. The supernatant was filtered with Amicon centrifugal filter (Millipore, Amicon Ultra-0.5 mL Centrifugal Filters, 10 kDa) for IBP concentration. The residue after the high-salt extraction was washed with TRIS buffer (50 mM TRIS pH 7.2, 50 mM NaCl, 0.05% Tween-20 and 1X protease inhibitor) and collected by centrifugation at 5000*g* for 10 min. To release covalently bound protein (CBP) from the cell wall fraction, we followed a modified method of Yi-Qin and Tsao (1985). The pellet was incubated with 0.5% cellulase and 2.5% macerozyme in 50 mM TRIS pH 7.2 with 1X protease inhibitor at 4 ºC for 24 h . After incubation, the mixture was centrifuged at 5000*g* for 10 min, then the supernatant was filtered with Amicon centrifugal filter for CBP concentration. IBP and CBP

fractions were stored at -20 ºC until it was used. The extracted proteins were used for subsequent analyses within a week.

Peroxidase activity assay

Mono-dimensional electrophoresis was carried out as semi-denaturing PAGE using 4% - 10% gradient gels (Bio-Red) at 4°C (Cesarino et al., 2012). Sample buffer was prepared without SDS, and protein (IBP or CBP) in sample buffer was loaded onto the gel without boiling. Since there is no SDS in sample buffer and protein is not denatured by boiling, peroxidase activity can be measured by guaiacol staining. Class III peroxidases were stained with 1% guaiacol and 0.03% H_2O_2 in 50mM sodium acetate buffer (pH 5.0), and when protein bands appeared as brown color, the reaction was stopped with distilled water and the gel was pictured. Then the gel was stained with Coomassie blue to visualize total protein.

Histochemistry

Wild-type and R2-4A seedlings were grown vertically for 6 days in continuous light. For comparison of hydrogen peroxide and superoxide level in roots, DAB (3, 3'-diaminobenzidine tetrahydrochloride, SIGMA D3939) and NBT (nitroblue tetrazolium, SIGMA N5514), respectively, were used. Lignin staining was performed using phloroglucinol (SIGMA P3502) in ethanol and HCl. The stock is 0.4g of phloroglucinol in 10 mL of 25% ethanol, and working solution is made with 230 μL of phloroglucinol stock, 230 μL of 25% ethanol, and 540 μL of 37% HCl. Tissue was stained with working solution at room temperature until magenta color appeared in mature vascular tissue. For cross section, 6-d-old seedlings were fixed with 4%

paraformaldehyde in PBS overnight, and embedded in LR White (EMS #14381). After sectioning, the specimens were stained with phloroglucinol solution.

To test the effect of hydrogen peroxide on ROS distribution in primary root, 3d-old seedlings of WT, *apy2* mutants, and R2-4A was treated with 30 μM H_2O_2 for 3d. Then the root length was measured with Image J, and roots was stained with DAB and NBT for comparison of hydrogen peroxide and superoxide level.

To observe auto-fluorescence of lignin, clearing the root and visualizing it were performed by using a described method (Hosmani et al., 2013). Briefly, 6-d-old seedlings were incubated with 0.24 M HCl and 20% methanol for 15 min at 57ºC, but WT seedlings were incubated at room temperature since their root tips were so fragile. Then, the solution was replaced with 7% NaOH and 60% ethanol for 15 min at room temperature. The seedlings were rehydrated by series of ethanol (40%, 20%, 10% and 5%) for 5 min each at room temperature after the clearing steps. Finally, seedlings were immersed in 25% glycerol and mounted on glass slides with 50% glycerol. Autofluorescence of lignin was observed with excitation 488mm (Leica DM IRE2 confocal microscope).

RESULTS

Confirmation of Microarray Data by qRT-PCR

Earlier work by Dr. Jian Wu compared transcript abundance differences between wild-type plants and RNAi mutants grown continuously in the estradiol inducer for 3.5 d in the dark and 6.0 d in the light using the NimbleGen Arabidopsis 4-Plex microarray, with 3 replicates. We anlazyed the data, and results revealed the identity of genes whose expression significantly changed (i.e.,

more than 2-fold, with false discovery rate of less than 5%) after apyrase suppression. Statistically significant changes in the transcript abundance of several hundred genes, including scores encoding wall/ECM-localized proteins, were observed. Control assays of wild-type plants treated with estradiol and single-knock-out mutants (apy1 or apy2) were conducted, and only expression changes that were not induced in wild-type plants by estradiol and were not induced in single-knock out mutants are described in this report. For 20 genes I confirmed the changes observed by microarray by qRT- PCR, and those gene changes persisted from 3.5 d to 6 d (Table 3.1). I also used qRT-PCR to show that for these 20 genes the transcript abundance changes observed at light-grown seedlings were also significant at dark-grown seedlings (Table 3.1) (Lim et al., 2014).

Apyrase Suppression Significantly Alters the Expression of Stress-related Genes and of Genes Encoding Proteins Expressed in the Extracellular Matrix

Genes that were significantly up- or down-regulated (> 2-fold; q value < 5%) were assessed by GO category analyses, and they were assigned a functional categorization by annotation for GO Cellular Component, GO Molecular Function and GO Biological Processes. The significant trends in GO Biological Processes showed that genes related to stress responses were the ones that most changed expression when APY1 and APY2 were suppressed (Fig. 3.1). Genes involved in response to abiotic/biotic stimulus and in developmental processes are the next most changed genes in R2-4A (Fig. 3.1). GO Cellular Component revealed that the second most changed expression genes encode proteins located mostly in the extracellular matrix (cell wall), and, somewhat less, in the nucleus. Among the gene families in the list of up-regulated genes in both categories of GO Cellular Component and GO Biological Processes, the most prominent are peroxidases (Table 3.2).

Apyrase Suppression Induces the Up-regulation of Type III Wall Peroxidases

Five Type III wall peroxidases that are co-expressed and up-regulated in roots undergoing abiotic stress (http://csbdb.mpimp-golm.mpg.de/csbdb/dbcor/ath/ath_tsgq.html) are all significantly up-regulated when APY1 and APY2 are suppressed in R2-4A mutants (Table 3.3). All are also strongly up-regulated as judged by microarray analysis, but two of them (AT5G05340 and AT5G06730) have q values higher than 5%. The genes encoding these 5 peroxidases all share common WRKY and other promoter motifs (Table 3.4). WRKY46 (AT2G46400) is significantly up-regulated in induced R2-4A mutants as judged by microarray analysis and qRT-PCR (Table 3.1).

We attempted to confirm whether the peroxidase gene expression changes observed by microarray and qRT-PCR were paralleled by equivalent changes in peroxidase cell wall proteins. To do this, cell wall proteins from the root of WT and R2-4A were extracted. Peroxidases from ionically bound proteins (IBPs) and covalently bound proteins (CBPs) in the cell wall were separated by gel electrophoresis, stained for peroxidase activity, then cut from the stained gel and identified by MALDI_TOF (Fig. 3.2). This approach identified only the most abundant and most extractable wall peroxidases, and none of those identified were among the five encoded by the genes identified in Table 3.3 that most changed expression as assayed by microarray and qRT-PCR data (Fig. 3.2 B and D). This suggested that those 5 peroxidases were not among those that most contributed to the peroxidase stained bands that were cut out for MALDI-TOF analysis, which were the only ones used for sequencing. Since the sequenced proteins were not total IBPs or CBPs, the approach we used could not identify all the peroxidases encoded by the genes up-regulated when APY1 and APY2 were suppressed in R2-4A.

Apyrase Suppression Induces the Unbalanced ROS level and Lignification in Roots

Since microarray data revealed that cell wall peroxidases are up-regulated in R2-4A, we tested how they function in R2-4A. Type III wall peroxidases use hydrogen peroxide (H_2O_2) substrates to induce protein cross-links and lignin formation in cell walls. As assayed by DAB (3, 3'-diaminobenzidine tetrahydrochloride) staining, the H_2O_2 level is increased toward the root tip in R2-4A roots since the elongation zone has disappeared (Fig. 3.3 A). After germination, wild-type roots maintain the same level of H_2O_2 from 2 d to 6 d, however R2-4A roots show increased H_2O_2 levels during their 3- to 6-d period of growth. They also accumulate lignin at this time (Fig. 3.4). Lignin is accumulated in secondary cell walls, such as in mature vascular tissue (Fig. 3.5 A and B). Normally lignin formation does not occur in the walls of cells in the meristematic, elongation, or early-differentiated cell zones of wild-type roots. However, R2-4A seedlings develop mature vascular tissue and lignification near their root tip (Fig 3.5 D), and accumulate lignin not only in vascular tissue, but also in the endodermis, cortex and epidermis (Fig. 3.4 B, D and F).

Maintenance of O_2^- levels is also important for normal cell growth (Dunand et al., 2006; Gapper and Dolan, 2006). Moreover, Tsukagoshi et al. (2010) showed that in normal root development, ROS distribution (the balanced distribution of H_2O_2 and O_2^-) is important for cell transition from proliferation to differentiation. The superoxide level is decreased in the meristematic zone that consists with decreased cell numbers in the zone of R2-4A (Fig. 3.3 B). Taken together, ROS distribution and lignification in roots are changed by apyrase suppression, resulting in increased lignification along with increased hydrogen peroxide level, and decreased superoxide level compared to wild type roots.

Mutants suppressed in neither *APY1* nor *APY2* singly do not show obvious phenotypic differences from wild-type plants, and two different RNAi lines have similar phenotypes

The expression level of cDNA with RT-PCR showed that *APY2* is expressed more in wild type plants than *APY1* (Fig. 3.6 A). Neither *apy1* nor *apy2* plants showed any obvious phenotype differences or growth inhibition compared to wild type plants (data not shown), so both have to be suppressed to have any effect on growth in *Arabidopsis*.

To further explore possible functional differences between *APY1* and *APY2* in growth control, we compared primary root growth in two different estradiol-inducible RNAi lines constructed by Jian Wu, R2-4A (which is null for *APY2* and knocked-down in *APY1* by estradiol) and R1-K4-5 (which is null for *APY1* and knocked-down in *APY2* by estradiol). Both RNAi lines inhibit same level of growth and show similar pattern of lignification in their primary roots after estradiol treatment (Fig. 3.6 B, C, and D).

Single and Double Knockouts of Wall Peroxidases and *Wrky46* in R2-4A Reduce the Growth Inhibition Induced by Apyrase Suppression in Roots

The microarray and qRT-PCR data suggested that 5 wall peroxidases help to mediate the inhibitory effects of apyrase suppression on primary root growth. To test this hypothesis I investigated whether single and multiple knockouts of 4 of these peroxidases altered primary root growth and whether those knockout lines could rescue the inhibition of root growth induced by apyrase suppression in R2-4A mutants.

Four knockout mutant lines of five peroxidases from microarray data were available on TAIR, and I used these to generate double and triple knockout mutant lines by crossing. Single

knockout lines (*per14*, *per49*, *per52*, and *per54*) and multiple knockout lines (*per14/49*, *per54/49*, and *per14/49/54*) showed significantly higher growth rates in their primary roots than wild type roots (Fig. 3.7 A). All those wall peroxidases share WRKY promoter motif (Table 3.4) and WRKY46 (AT2G46400) is significantly up-regulated in R2-4A (Table 3.1), so the root growth of *wrky46* was also compared with WT. The rate of root growth in *wrky46* was similar with other mutant lines of wall peroxidases (Fig. 3.7 A).

Next question was whether those knockout lines could rescue the inhibition of root growth under apyrase suppression or not. Thus I crossed R2-4A with those mutant lines, and three lines (*per54* + R2-4A, *per54/49* + R2-4A, and *wrky46* + R2-4A) were produced. The data revealed that knockouts of wall peroxidases and *wrky46* in R2-4A could partially reduce the growth inhibition induced by apyrase suppression (Fig. 3.7 B). As judged by staining assays after estradiol induction, two lines, *per54/49*+R2-4A and *wrky46* + R2-4A, had reduced lignification and altered unbalanced ROS levels compared to R2-4A lines (Fig. 3.8). Moreover, the up-regulation of *PER54* was much less in *wrky46* + R2-4A, but the up-regulation of *PER14* and *PER49* was equivalent to that in R2-4A. The expression of these 3 peroxidases was not changed in *wrky46* compared to Col-0 WT seedlings (Table 3.5).

DISCUSSION

The significant root growth inhibition and root morphology changes observed in the primary roots of seedlings suppressed in APY1 and APY2 expression (Wu et al., 2007) are reminiscent of the growth inhibition effects observed in seedlings responding to biotic and abiotic stresses. Many different stresses can induce similar responses in plants, the so-called stress-

induced morphogenic responses (SIMRs) (Potters et al., 2009). The SIMRs in roots involve common physiological processes such as altered ROS production, hormone transport changes, inhibition of elongation and consequent formation of root hairs near the apex, and blocked cell division in the primary meristem (Potters et al., 2007). All of these changes have been documented in the roots of estradiol-induced R2-4A seedlings (Wu et al., 2007; Liu et al., 2012), so it is not surprising that the gene expression changes in these mutants would be characteristic of plants subjected to stress conditions.

The striking finding in our GO analyses is that although the R2-4A seedlings were grown under sterile conditions, their gene expression changes in response to apyrase suppression remarkably resembled the transcriptomic changes plants undergo when under abiotic and/or biotic stress (Fig. 3.1; Lim et al., 2014). One possible explanation for this is that both stress conditions and suppression of *APY1/APY2* expression release ATP into the ECM. The increased [eATP] resulting from apyrase activity inhibition can occur within minutes (Wu et al., 2007), and the release of ATP from wounded cells or cells rendered leaky by pathogen attack would also be expected to occur within minutes. The signaling changes induced by eATP, which include increased $[Ca^{2+}]_{cyt,}$ increased NO and ROS production, and increased MAP kinase expression (Tanaka et al., 2010; Clark and Roux, 2011; Choi et al., 2014). The fact that SIMRs in roots are related to altered ROS production (Apel and Hirt 2004; Pierik et al., 2006; Romera and Alcantara, 1994; Schmidt and Bartels, 1996; Schmidt et al., 2000; Zolla et al., 2010) is further supporting a functional connection between increased eATP and the induction of altered growth and morphology changes characteristic of stress responses.

Although one might expect that the signaling pathways linking different hormone treatments to growth changes would converge on similar gene expression changes, Nemhauser et

al. (2006) found that the transcript abundance changes induced respectively by ABA, auxin, brassinosteroid, cytokinin, ethylene, gibberellin, and jasmonates were remarkably dissimilar from each other. They concluded that different hormones regulate growth and other responses in plants by distinctly different networks of genes. The transcriptional changes that link apyrase suppression to growth inhibition are also distinct from the transcriptional changes that occur in response to growth-inhibitory levels of any one of the hormones. Statistical techniques similar to Nemhauser et al. (2006) revealed that the overall pattern of gene expression changes induced by apyrase suppression is different from patterns observed when auxin, ethylene, ABA, or brassinosteroids inhibit growth (data not shown).

Superoxide (O_2^-) is rapidly converted to H_2O_2 in cells, and peroxidases use H_2O_2 as a substrate to catalyze diverse products that affect root growth (Dunand et al., 2007; Gapper and Dolan, 2006; Nakagami et al., 2006; Tsukagoshi, 2012; Tsukagoshi et al., 2010). Because the inhibition of primary root growth and up-regulation of several Type III wall peroxidases occur together in roots of estradiol-induced R2-4A mutants, we assayed ROS levels in these roots. With estradiol treatment the balance of O_2^- and H_2O_2 levels in the R2-4A primary root starts to change near when apyrase suppression peaks (~ 3 d), and by 6 d, the level of H_2O_2 is much higher than that of O_2^- (Fig. 3.3). Maintenance of O_2^- levels is important for normal cell growth (Dunand and Penel, 2007; Gapper and Dolan, 2006), and when root H_2O_2 levels are increased, the progression of meristematic cells through S-phase and M-phase is decreased (Tsukagoshi, 2012). Thus, unbalanced those two ROS in R2-4A roots induces cell division and growth.

Since ROS redistribution is upstream of cell wall changes, and apyrase suppression induced an increased H_2O_2, I tested the effect of H_2O_2 on ROS distribution in primary roots. Both WT and R2-4A seedlings treated with H_2O_2 showed unbalanced ROS distribution compared to

WT without the treatment (Fig. 3.9). Also those roots treated with H_2O_2 showed significant growth inhibition. Interestingly, the growth of H_2O_2-treated roots of *apy2* single mutants was not significantly different from that of the roots of induced R2-4A (data not shown) mutants, although the morphology of epidermal cells near root tip was not similar. This suggests that the growth effects and epidermal morphology effects (cell bulging) are distinct and do not depend on each other.

Along with increased H_2O_2 in roots, there is an increased abundance of transcripts encoding Type III peroxidases (Table 3.3) that can catalyze a broad range of physiological processes like crosslinking of phenolic compounds to proteins and polysaccharides, and lignin deposition (Passardi et al., 2004; Passardi et al., 2005; Almagro et al., 2009; Djebali et al., 2011; Zhang et al., 2012). Peroxidase genes are up-regulated by plants in response to biotic stresses, and their expression is associated with increased resistance to insect herbivores and pathogens (Hu et al., 2012; Suzuki et al., 2012). Five of the genes most up-regulated when *APY1* and *2* are suppressed encode Type III wall peroxidases that are co-expressed in roots and up-regulated together by abiotic stresses known to inhibit root growth. The genes encoding these 5 peroxidases all share common WRKY motifs (Table 3.4), so it is noteworthy that WRKY46 (AT2G46400), whose expression enhances basal resistance against *Pseudomonas* pathogens (Hu et al., 2012), is also significantly up-regulated in induced R2-4A mutants. Clearly, peroxidase up-regulation occurs in plant responses to both biotic and abiotic stresses, so increased wall peroxidase expression could serve as a key convergence point in the signaling pathways that crossover in stress signaling networks (Fujita et al., 2006). The up-regulation of peroxidases is often correlated with growth inhibition through peroxidase-catalyzed wall cross-linking reactions, lignin formation, and/or the ROS changes induced by these enzymes (Pedreira et al., 2011). We confirmed that the apyrase-

suppressed mutants have much more extensive lignin formation than wild-type plants, and this difference continues to increase with time (Figs. 3.4 B, D and F). Taken together, increased H_2O_2 level, decreased O_2^- level, increased expression of wall peroxidases, and increased lignin and boron accumulation in cell walls would all contribute to root growth inhibition by gradually decreasing the number and size of cells in meristematic and elongation zones.

Since wall peroxidases play an important role in cell growth control, it was reasonable to postulate that reduced expression of the peroxidases could rescue the growth inhibition caused by apyrase suppression. To test this hypothesis, single and double knockouts of wall peroxidases were crossed with R2-4A. Two of the crossed lines, *per54* + R2-4A and *per54/49* + R2-4A, reduced by more than 10% the effect of apyrase suppression on root growth inhibition after estradiol treatment (Fig. 3.7 B). It was less obvious, however, that reduced expression of wall peroxidases changed the unbalanced ROS distribution and lignification in R2-4A roots (Fig. 3.8). This could be due to the fact that there are a number of wall peroxidases that could replace the function of the peroxidases that were knocked out. The changed ROS level and lignin content we did observe with staining methods would have to be confirmed by more sensitive and more specific chemical assays.

WRKY46 is one of the major transcription factors (TF) controlling the expression of peroxidases, and it is localized in the stele of root apices (Ding et al., 2013), thus we tested whether this TF is essential to transduce the suppression of apyrase into suppression of primary root growth. The null line, *wrky46*, was crossed with R2-4A, and the *wrky46* + R2-4A mutant, like *per54* + R2-4A and *per54/49* + R2-4A, partially reversed the root growth inhibition observed in R2-4A mutants after estradiol induction (Fig. 3.7 and 3.8). However, although the up-regulation of PER54 was reduced in *wrky46* + R2-4A, that of PER14 and PER 49 were essentially unchanged, i.e., those expressions were both up-regulated as much as in R2-4A (Table 3.5). Even though all those wall

peroxidases have W-box promoter motif for WRKY TF binding and WRKY46 is a key regulatory participant in abiotic and biotic stress responses (Ding et al., 2013; Hu et al., 2012), this result implies that WRKY46 is not the main TF for most of the peroxidases that control primary root growth.

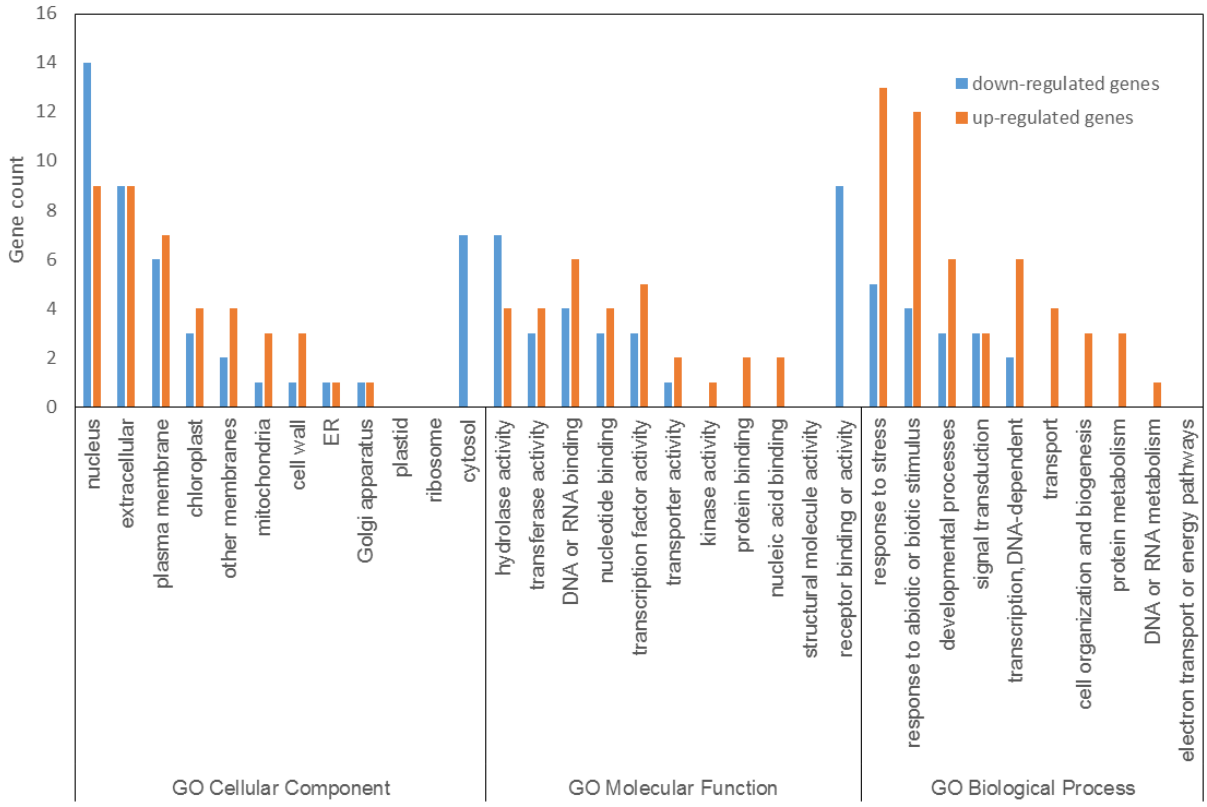
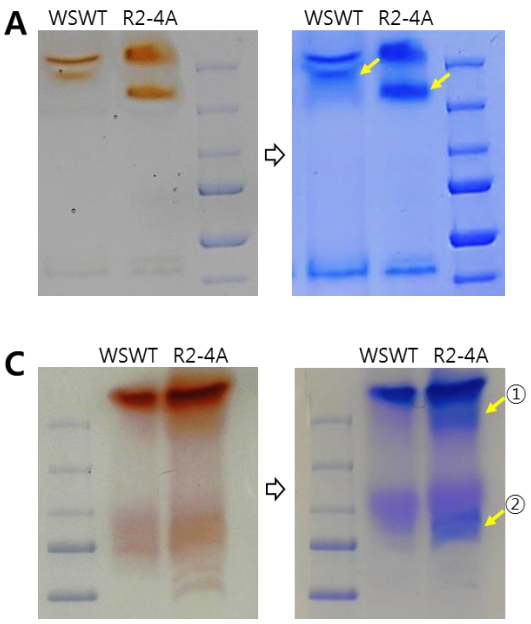


Figure 3.1. GO categories characterization of differentially expressed genes. Up-regulated and down-regulated overlap genes in 3d-dark and 6d light-grown seedlings. The criteria of differentially expressed genes is FDR q-values less than 5% and at least 2-fold change of gene expression. The value of Y axis represents number of genes.



B

Identified Proteins	Accession Number	M.W.	fold changes
Peroxidase 22	P24102	38 kDa	1.03
Peroxidase 32	Q9LHB9	39 kDa	0.54
Peroxidase 37	Q9LDN9	38 kDa	0.13
Peroxidase 54	Q9FG34	37 kDa	only in R2-4A

D

Identified Proteins	Accession Number	M.W.	①	②
Peroxidase 42	Q9SB81	37 kDa		v
Peroxidase 54	Q9FG34	37 kDa	v	

Figure 3.2. Peroxidases activity assay with extracted wall proteins. Ionically bound proteins (IBPs) (A) and covalently bound protein (CBPs) (C) in cell wall are separated and stained in SDS-PAGE gel for peroxidases activity assay. Peroxidases in the gel stained as brown color with 1% guaiacol and 0.03% H_2O_2 in 50mM Na-acetate (sodium acetate, NaOAc) buffer (pH 4.6), then the gel stained with coomassie blue for total protein staining. (B) The peroxidases that are differently separated between WT and R2-4A (yellow arrows) in IBPs are identified with protein sequencing. (D) Strongly stained peroxidases in CBPs of R2-4A are identified with protein sequencing.

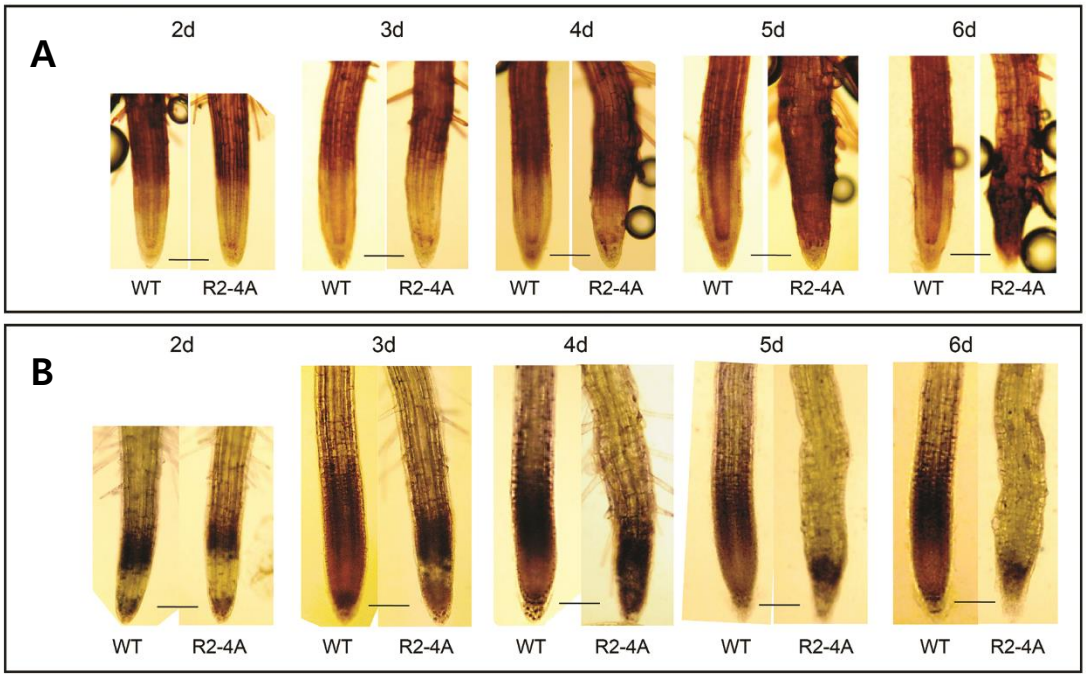


Figure 3.3. Higher ROS levels are induced R2-4A roots when apy1 and apy2 are suppressed. (A) Hydrogen peroxide levels in wild-type (WT) and R2- 4A roots from 2 d to 6 d after estradiol treatment. DAB (3, 3'-diaminobenzidine tetrahydrochloride) was used to stain hydrogen peroxide. (B) Superoxide levels in WT and R2-4A roots from 2 d to 6 d. NBT (nitroblue tetrazolium) was used to stain superoxide. Images are representative of at least three biological repeats. Scale bar = 100 μm.

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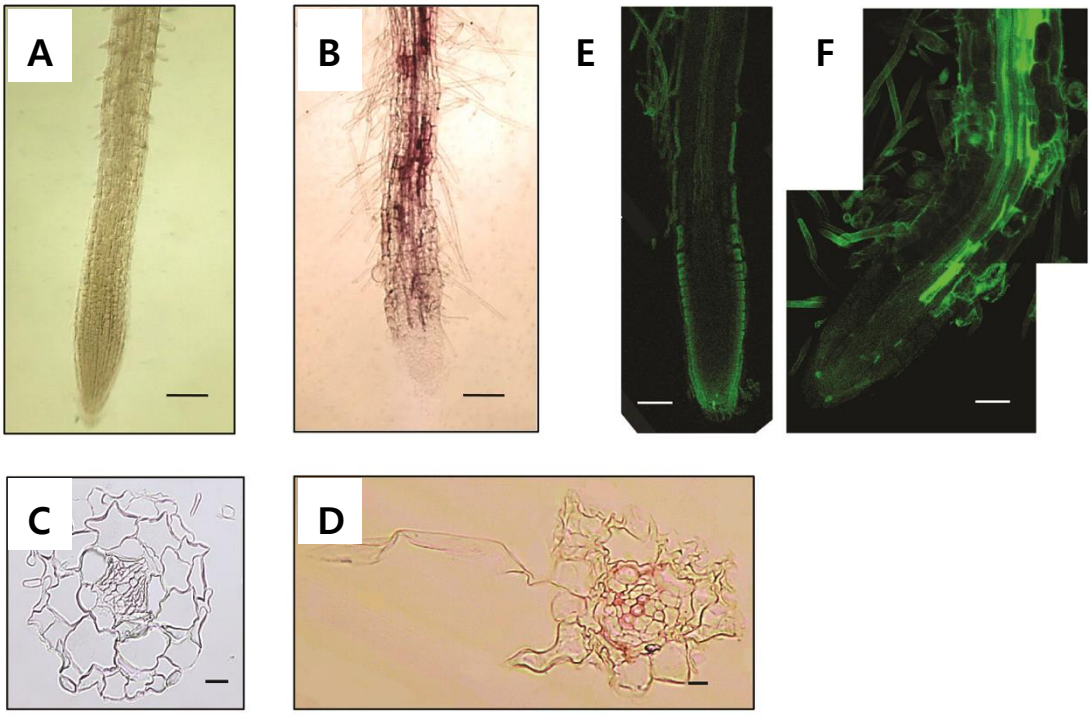

Figure 3.4. Higher Lignin Levels Are Induced in R2-4A Roots When APY1 and APY2 Are Suppressed. Lignin staining of 6-d-old roots with phloroglucinol in (A) wild-type (WT) and (B) R2-4A roots. Magenta color indicates lignin. Cross sections of (C) WT and (D) R2-4A roots stained with phloroglucinol. Cross sections are from region 200~600 μm from root tip where vascular tissue is not fully matured in WT. Lignin auto-fluorescence of WT (E) and R2-4A (F) are showing in longitudinal section with confocal microscope. Images are representative of at least three biological repeats. Scale bar = 100 μm in (A) and (B), 10 μm in (C) and (D), and 50 μm in (E) and (F).

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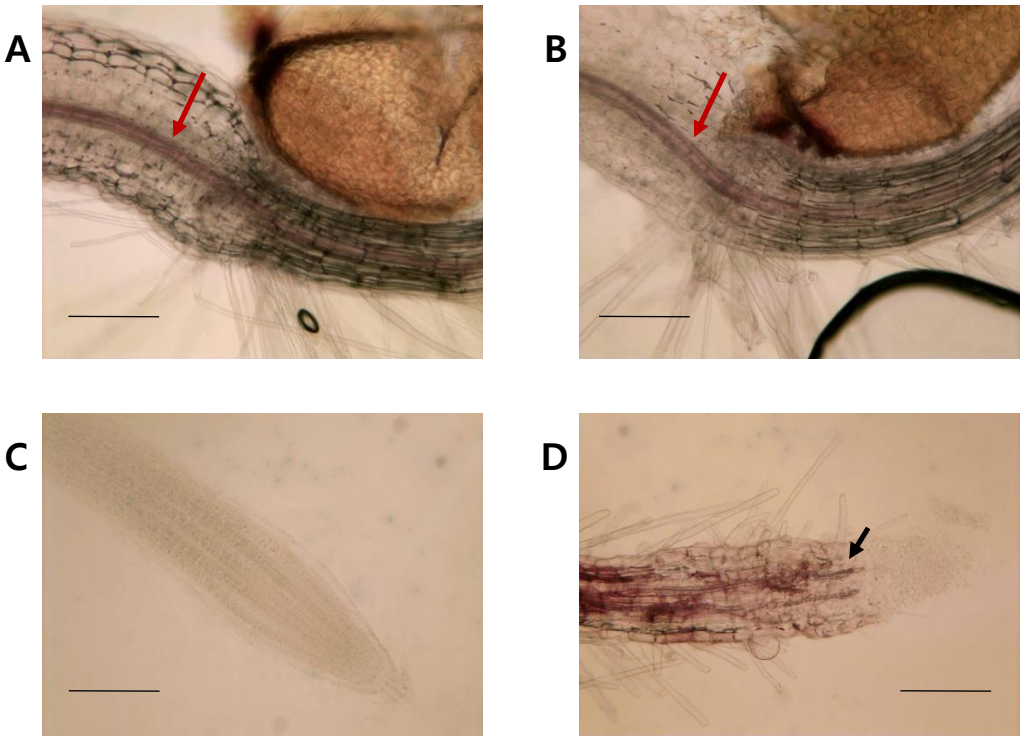

Figure 3.5. Vascular tissue in wild-type and R2-4A. The structure and lignin formation of vascular tissue (red arrows) in hypocotyl and upper root are similar between wild-type (A) and R2-4A (B). However, R2-4A shows matured vascular tissue near root tip (D; black arrow) while there is no vascular tissue in root tip of wild-type (C). Magenta color indicates lignin stained with phloroglucinol. Scale bar=100µm

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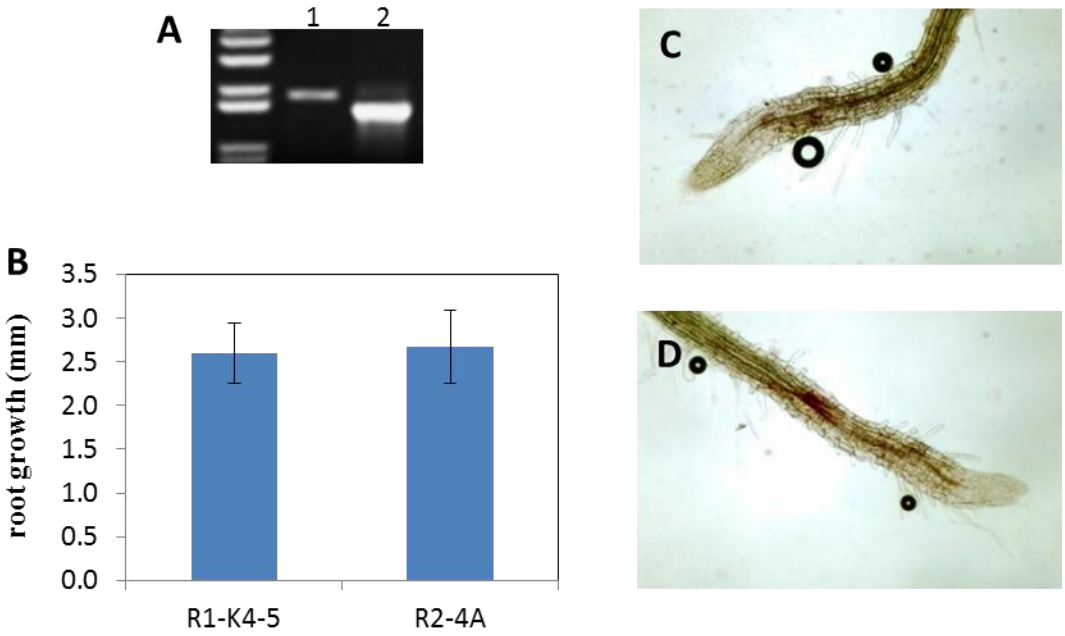

Figure 3.6. Phenotype comparison between R1-K4-5 and R2-4A. A, mRNA expression levels of *APY1* (1: 1.85kb) and *APY2* (2: 1.53kb) in WT. B, Comparison of 6-d-old root growth with estradiol treatment between R1-K4-5 and R2-4A. C and D, lignin staining with phloroglucinol of R1-K4-5 (C) and R2-4A (D).

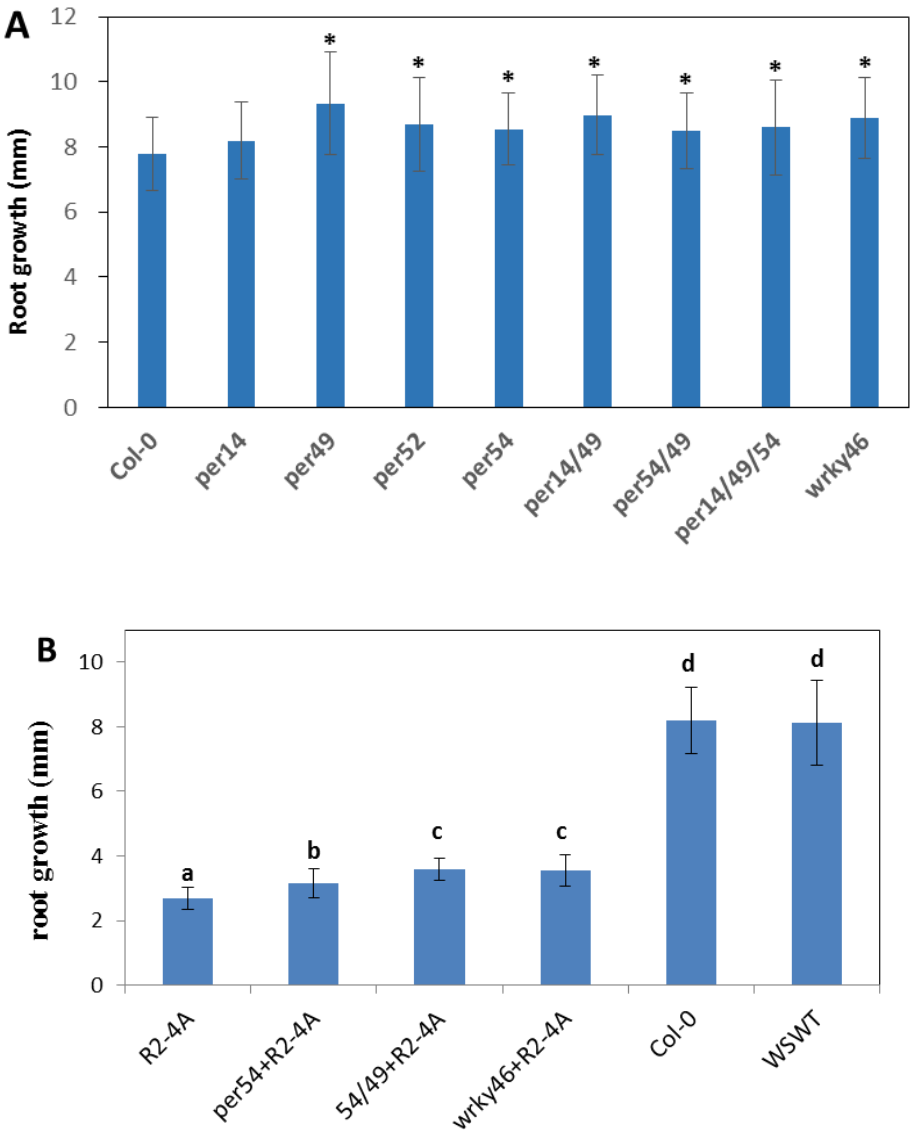

Figure 3.7. The effect of gene expression of wall peroxidases and *WRKY46* on root growth. A, Root growth of Col-0 and peroxidase KOs (single, double and triple KOs) for 3 days from 3d-old (* p value ≤ 0.001 compared to Col-0, n ≥ 50). B, Knockout lines of wall peroxidases and wrky46 in R2-4A reduce the growth inhibition by apyrase suppression. Comparison of R2-4A root growth with WT and other mutants. WT and all mutants showed significantly longer roots than R2-4A (b~d: p value ≤ 2.03E-07 compared to a; n ≥ 70). Error bar indicates standard deviation.

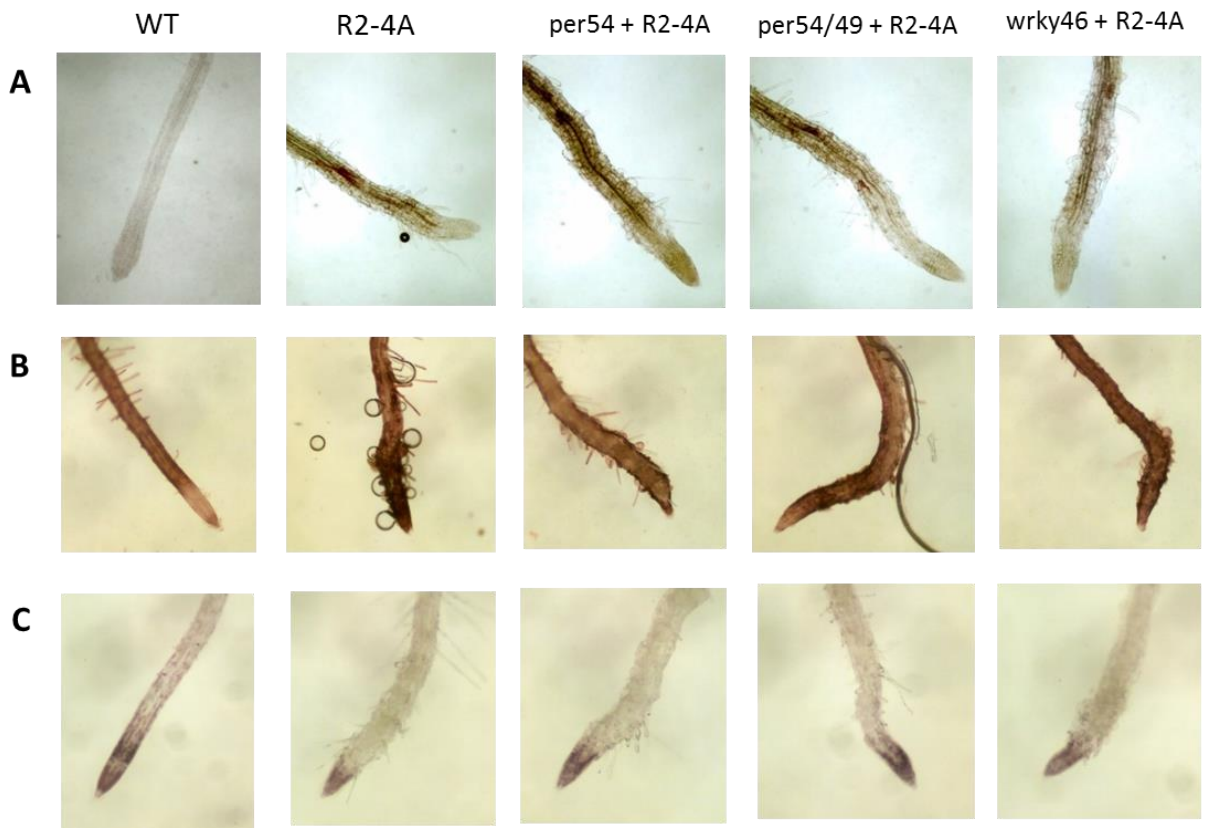

Figure 3.8. Comparison of lignin accumulation and ROS level in roots of WT with mutant lines. A, lignin staininig. B, Hydrogen peroxide staining. C, superoxide staining

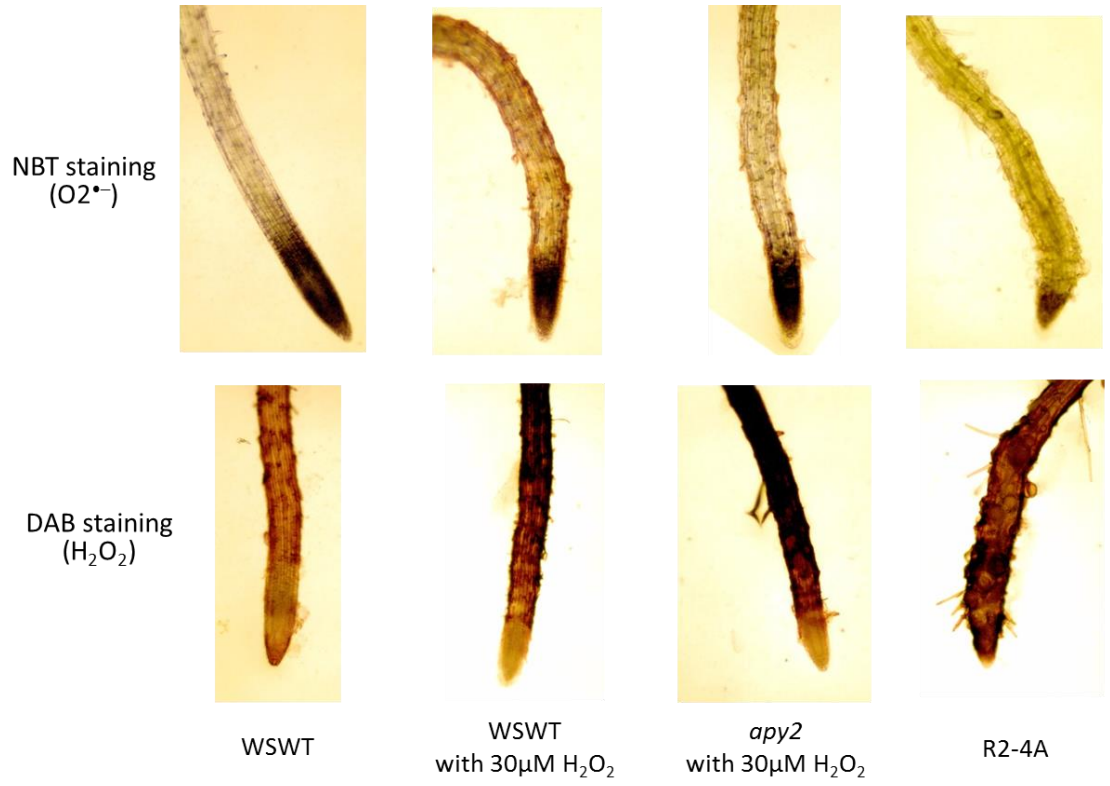

Figure 3.9. The effect of hydrogen peroxide on ROS distribution in primary root. The response of WT and *apy2* mutants to applied H_2O_2 is similar, but R2-4A showed a more severe unbalanced ROS distribution. 30 μM H_2O_2 was applied to 3d-old seedlings for 6d. DAB (3, 3'-diaminobenzidine tetrahydrochloride) was used to stain hydrogen peroxide. NBT (nitroblue tetrazolium) was used to stain superoxide. Images are representative of at least three biological repeats.

Genes	Dark		Light	
	3.5d	6d	3.5d	6d
AT1G05680 IAA b glucosyl & UDP glucosyl transferase	0.83 (0.28)	0.36	0.77	0.82 (0.48)
AT1G07160 PP2c phosphatase	0.89 (0.42)	0.59	1.2	0.62 (0.41)
AT1G21250 cell wall-associated kinase (WAK1)	6.56 (2.02)	1.02	3.16	1.52 (4.24)
AT1G73330 protease inhibitor	0.38 (0.5)	0.51	0.78	0.38 (0.24)
AT2G18150 Peroxidase (PER 15) ECM	4.53 (9.94)	5.74	5.15	18.9 (21.6)
AT2G31083 Clavata3	0.06 (0.06)	0.01	0.13	0.02 (0.39)
AT2G33790 Arabinogalactan protein 30 (APG30)	0.26 (0.05)	0	0.08	0 (0.2)
AT2G37440 phosphatidylinositol phosphatase	0.42 (0.31)	0.42	0.59	0.43 (0.24)
AT2G38340 ERF/AP2 TF; DREB subfamily	1.45 (3.73)	4.46	2.23	2.02 (7.54)
AT2G46400 WRKY46	4.26 (2.72)	2.68	4.53	5.75 (2.09)
AT3G28860 MDR1;PGP19	1.11 (1.13)	0.96	0.9	1.22 (1.23)
AT3G56980 BHLH039/ORG3	0.59 (0.17)	0.35	0.86	2.59 (0.18)
AT4G15290 similar to cellulose synthase	0.28 (0.42)	0.17	0.1	0.04 (0.3)
AT4G17030 expansin-related (NPA induced)	1.19 (2.32)	2	1.8	2.53 (2.05)
AT4G22950 AGAMOUS-like 19 TF	0.78 (3.71)	3.4	0.35	2.43 (1.44)
AT4G23590 aminotransferase	0.99 (0.43)	0.16	2.01	0.56 (0.37)
AT4G37160 SKS15; pectinesterase-like	0.33 (0.3)	0.06	0.43	0.09 (0.3)
AT5G06720 Peroxidase 53	4.54 (6)	4.77	5.03	7.95 (2.43)
AT5G15380 DRM1	4.75 (5.25)	8.45	9.92	7.64 (4.45)
AT5G64060 ANAC103	6.74 (2.38)	5.06	5.83	6.47 (4.67)

Table 3.1. qRT-PCR Assays Confirm Microarray data. qRT-PCR assays of 20 selected genes whose transcript abundance changed significantly in R2-4A seedlings after the suppression of APY1 and APY2 were assayed at 3.5 and 6.0 d. Most gene expression changes persist from 3.5 to 6 d in dark and light. Numbers in parentheses are the NimbleGen microarray values for fold-changes, which were measured in 3.5d-dark- and 6.0d-old light-grown seedlings (performed by Jian Wu and Jianchao Yao). *Numbers represent average fold changes, and all qRT-PCR numbers represent average fold changes of more than three biological repeats.

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A. Up-regulated genes overlapped between 3.5d Dark and 6d light

GO biological process	Gene name	Located in
response to stress		
AT1G19610.1	LOW-MOLECULAR-WEIGHT CYSTEINE-RICH 78 (LCR78)	Extracellular
AT1G21250.1	CELL WALL-ASSOCIATED KINASE 1 (WAK1)	Extracellular, plasma membrane, vacuole
AT1G21520.1	unknown protein	endoplasmic reticulum
AT1G49860.1	GLUTATHIONE S-TRANSFERASE 14 (GSTF14)	cytoplasm
AT2G18150.1	PEROXIDASE 15	cell wall, extracellular region
AT2G38340.1	DEHYDRATION RESPONSE ELEMENT-BINDING PROTEIN 19 (DREB19)	nucleus
AT2G46400.1	WRKY DNA-binding protein 46 (WRKY46)	nucleus
AT3G47480.1	Calcium-binding EF-hand family protein	plasma membrane
AT3G50930.1	CYTOCHROME BC1 SYNTHESIS (BCS1)	plasma membrane
AT4G36430.1	PEROXIDASE 49	cell wall, extracellular region
AT4G39830.1	Cupredoxin superfamily protein	Extracellular
AT5G06720.1	PEROXIDASE 53	Golgi apparatus, extracellular region
AT5G15380.1	Domains rearranged methylase 1 (DRM1)	Nucleus
response to abiotic and biotic stimulus		
AT1G06520.1	GLYCEROL-3-PHOSPHATE SN-2-ACYLTRANSFERASE 1 (GPAT1)	membrane, mitochondrion
AT1G21250.1	CELL WALL-ASSOCIATED KINASE 1 (WAK1)	Extracellular, plasma membrane
AT1G49860.1	GLUTATHIONE S-TRANSFERASE 14 (GSTF14)	cytoplasm
AT2G18150.1	PEROXIDASE 15	cell wall, extracellular region
AT2G38340.1	DEHYDRATION RESPONSE ELEMENT-BINDING PROTEIN 19 (DREB19)	nucleus
AT2G46400.1	WRKY DNA-binding protein 46 (WRKY46)	nucleus
AT3G47480.1	Calcium-binding EF-hand family protein	plasma membrane
AT3G50930.1	CYTOCHROME BC1 SYNTHESIS (BCS1)	plasma membrane
AT4G36430.1	PEROXIDASE 49	cell wall, extracellular region
AT4G39830.1	Cupredoxin superfamily protein	Extracellular
AT5G06720.1	PEROXIDASE 53	Golgi apparatus, extracellular region
AT5G15380.1	Domains rearranged methylase 1 (DRM1)	nucleus
Developmental process		
AT1G06520.1	GLYCEROL-3-PHOSPHATE SN-2-ACYLTRANSFERASE 1 (GPAT1)	membrane, mitochondrion
AT1G49860.1	GLUTATHIONE S-TRANSFERASE 14 (GSTF14)	Cytoplasm
AT3G01600.1	NAC DOMAIN CONTAINING PROTEIN 44 (NAC044)	nucleus
AT5G06720.1	PEROXIDASE 53	Golgi apparatus, extracellular region
AT5G55490.1	GAMETE EXPRESSED PROTEIN 1 (GEX1)	plasma membrane
AT5G64060.1	NAC domain containing protein 103 (NAC103)	nucleus

Table 3.2. The list of gene expression changed in both 3.5d Dark and 6d light.

B. Down-regulated genes overlapped between 3.5d Dark and 6d light

GO biological process	Gene name	located in
response to stress		
AT1G05680	UDP-glucosyltransferase (UGT74E2)	cellular_component
AT1G14960	Polyketide cyclase/dehydrase and lipid transport superfamily protein	nucleus
AT1G51470	BETA GLUCOSIDASE 35 (BGLU35)	extracellular region
AT1G73330	DROUGHT-REPRESSED 4 (DR4)	extracellular region
AT2G01520	MLP-LIKE PROTEIN 328 (MLP328)	nucleus
AT2G39040	Peroxidase 24	extracellular region
AT4G11210	Disease resistance-responsive (dirigent-like protein) family protein	extracellular region
AT4G14060	Polyketide cyclase/dehydrase and lipid transport superfamily protein	nucleus
AT5G04150	basic helix-loop-helix transcription factor family protein (BHLH101)	nucleus
AT5G47450	ARABIDOPSIS THALIANA TONOPLAST INTRINSIC PROTEIN 2;3 (ATTIP2;3)	membrane
AT5G59090	subtilase 4.12 (SBT4.12)	nucleus
response to abiotic or biotic stimulus		
AT1G05680	URIDINE DIPHOSPHATE GLYCOSYLTRANSFERASE 74E2 (UGT74E2)	cellular_component
AT1G51470	BETA GLUCOSIDASE 35 (BGLU35)	extracellular region
AT1G73330	DROUGHT-REPRESSED 4 (DR4)	extracellular region
AT3G45680	Major facilitator superfamily protein	membrane
AT5G47450	ARABIDOPSIS THALIANA TONOPLAST INTRINSIC PROTEIN 2;3 (ATTIP2;3)	membrane
Developmental processes		
AT1G05680	UDP-glucosyltransferase (UGT74E2)	cellular_component
AT1G63450	XYLOGLUCAN-SPECIFIC GALACTURONOSYLTRANSFERASE 1 (XUT1)	Golgi apparatus, membrane
AT2G01520	MLP-LIKE PROTEIN 328 (MLP328)	cytosol, nucleus
AT4G15290	CELLULOSE SYNTHASE LIKE 5 (CSLB05)	Golgi apparatus, membrane
AT5G47980	HXXXD-type acyl-transferase family protein	cytoplasm

Table 3.2. The list of gene expression changed in both 3.5d Dark and 6d light.

Microarray data	3.5d Dark	6d Light
AT2G18140 Peroxidase (PER 14)	2.50	1.51
AT4G36430 Peroxidase (PER 49)	10.85	11.22
AT5G05340 Peroxidase (PER 52)	10.01	13.19
AT5G06720 Peroxidase (PER 53)	6.00	2.43
AT5G06730 Peroxidase (PER 54)	5.33	3.5

Table 3.3. Transcript abundance of five genes encoding type III wall peroxidases in whole seedlings increases in R2-4A mutants suppressed in their expression of apy1 and apy2. The fold changes for each gene from microarray analysis are presented.

Motifs	Binding Site Sequence	Transcription Factor Family	Function
W-box promoter	TTGAC(C/T)	WRKY	biotic and abiotic stress responses[1]
MYB4 binding site	A(A/C)C(A/T)A(A/C)C	MYB	response to environmental stresses[2]
MADS motif	CC(A/T)6GG (CArG motif)	MADS domain protein AGL15	all major aspects of development[3]
MYB1AT	AACCA(A/T)	MYB	response to water stress and ABA[4]

Table 3.4. Common promoter motifs in 5 wall peroxidases (AT2G18150; AT4G36430; AT5G05340; AT5G06720; AT5G06730) that are up-regulated in R2-4A mutants suppressed in APY1 and APY2 expression.

[1] Rushton, Paul. "The Lab of Dr. Paul Rushton". wordpress.com.

[2] Plant Cell 14(3): 559-74

[3] Plant Cell 15: 63-78

[4] J Biol Chem.278: 28154-28159

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	Peroxidase 14	Peroxidase 49	peroxidase54	control
R2-4A	22.577	26.65	22.826	WSWT
wrky46	0.89	0.806	1.262	Col-0
wrky46 + R2-4A	13.704	54.176	6.364	Col-0
wrky46 + R2-4A	25.757	25.883	9.128	WSWT
R1-K4-5	19.459	17.852	3.567	WSWT

Table 3.5. Peroxidase expression in 6-d-old light grown seedlings of *wrky46* mutant lines. All qRT-PCR numbers represent average fold changes of more than three biological repeats of mutant lines compared to WT plants. *wrky46* background is Col-0, and R2-4A background is WS.

Chapter 4. Conclusion

The [eATP] is increased when the Arabidopsis apyrases APY1 and APY2 are suppressed (Wu et al., 2007; Lim et al., 2014), and this indirect evidence suggests that these apyrases function in part as ectoapyrases to regulate eATP levels outside the cell, which helps maintain optimal eATP levels for normal plant growth (Wolf et al., 2007). As described in the hypothetical model proposed in Figure 4.1, increased [eATP] induces numerous signaling changes beginning with increased cytosolic Ca^{2+} levels (Demidchik et al. 2003, 2009, 2011; Jeter et al. 2004) and increased ROS via the NADPH oxidases in *Arabidopsis* (Song et al. 2006; Clark et al., 2010; Clark et al., 2011). Application of a major ROS, H_2O_2, to *Arabidopsis* root epidermis induces Ca^{2+} influx, and this transient is greater in the elongation zone of primary roots than in the mature epidermis (Demidchik et al., 2007). This application also activates K^+ efflux in root cells, and this loss of potassium plays a key role in inducing programmed cell death (Demidchik et al., 2010). Moreover, maintenance of another ROS, O_2^-, in roots is important for normal cell growth (Chen et al., 1999; Dunand et al., 2007; Gapper and Dolan, 2006). In this dissertation I report that the H_2O_2 level is increased and O_2^- level is decreased in R2-4A roots compared to WT plant roots when apyrase suppression results in increased [eATP]. These data could help explain why reduced cell growth in the elongation zone and the meristematic zone are the most evident phenotypic changes induced by apyrase suppression in R2-4A roots.

The higher level of extracellular nucleotides that result from APY1/APY2 suppression also has an effect on hormones involved in plant growth. First, Tang et al. (2003) revealed that applied nucleotides disrupt polar auxin transport in *Arabidopsis* roots. This finding led us to hypothesize that increased [eATP] induced by apyrase suppression could also disrupt polar auxin transport in

Arabidopsis roots. Research carried out by our collaborators demonstrated that both in hypocotyls and roots, reduction in apyrase expression using RNAi leads to reductions in basipetal auxin transport in those tissues in which growth is altered by apyrase suppression (Liu et al., 2012). Correspondingly, I demonstrated that an altered GFP reporter pattern occurs in both DR5:GFP-expressing roots of estradiol-treated R2-4A mutants and of WT roots treated with apyrase inhibitor compared to untreated DR5:GFP-expressing roots of WT seedlings. Additionally, estradiol-treated R2-4A seedlings present similar root phenotypes, reduced lateral root formation (Wu et al., 2007) and reduced acropetal IAA transport (Liu et al., 2012), as mutants in genes encoding auxin transport proteins (Marchant et al., 2002; Benková et al., 2003; Lewis et al., 2011) and wild-type plants treated with inhibitors of polar auxin transport (Reed et al., 1998; Casimiro et al., 2001). All these findings suggest that the root growth suppression by *APY* mRNA interference, including diminished rates of both cell elongation and mitosis, is at least partially due to auxin transport inhibition.

A second hormonal effect of increased levels of the applied nucleotides is to induce a rapid increase in the transcript abundance of genes encoding ACC Synthase6 and Ethylene Response Factor4 in *Arabidopsis* (Jeter et al., 2004; Song et al., 2006). Correspondingly, higher levels of applied nucleotides can induce ethylene production in the cotton fibers of cultured ovules, and the ACC synthesis inhibitor aminovinyl-Gly can block the effects of applied ATPγS and ADPβS on the growth of fibers (Clark et al., 2010a), hypocotyls (Butterfield and Roux, unpublished; Roux and Steinebrunner, 2007), and root hairs (Clark and Roux, unpublished). One of the abnormal phenotypes observed in the primary roots of induced R2-4A mutants, the swelling and blebbing of epidermal cells, is induced by ACC (Tsang et al., 2011). Thus, it is likely that in addition to auxin

transport changes, there is also a role for ACC and/or ethylene in inducing the root growth and development changes observed in the R2-4A root phenotype.

One mechanism by which APY1/APY2 suppression could block auxin transport would be by suppressing the transcript abundance of genes that are involved in auxin transport. So, I compared mRNA levels for auxin transporters/carriers in both aerial and root tissues of WT and R2-4A in time course after estradiol treatment. However, there were no expression changes of those genes induced by apyrase suppression. These results do not rule out the possibility that apyrase suppression could alter the posttranscriptional activity of auxin transporters.

Wall peroxidases can catalyze the oxidative degradation of auxin (Krylov and Dunford, 1996; Gazarian et al., 1998; Potters et al., 2009), and, by promoting increased lignification of cell walls in the Casparian strip, they could alter auxin transport which passes through this region in primary roots (Naseer et al., 2012). It seems likely that these wall changes are kinetically downstream of the NO and ROS changes that take place in less than 30 min when they are induced by increased [eATP] (Salmi et al., 2013). As mentioned in chapter 3, increased expression of wall peroxidases, unbalanced ROS distribution and abnormal lignification are significantly induced by suppression of apyrases in primary root.

The Casparian strip has lignin in the cell walls of adjacent endodermal cells to form a tight junction in the differentiation zone, and this junction blocks extracellular diffusion of ions and other charged materials across the endodermis (Hosmani et al., 2013). In addition to the abnormal accumulation of lignin in root cells, induced R2-4A mutants form the Casparian strip between endodermis cells near their root tip, which does not happen in this region of WT roots (Fig. 3.4 F). This change is consistent with disappearance of the elongation zone in R2-4A roots, and it could contribute to the physical disruption of polar auxin transport in the mutant roots.

Remaining questions

In Figure 4.1 I note remaining questions about whether DORN1 is the receptor for eATP-induced changes in the signaling events leading to increased wall lignification and how increased ROS leads to gene expression changes, such as increased expression of genes encoding wall peroxidases. I discuss here additional questions related to other wall changes that could be mediated by increased ROS and wall peroxidase activity.

Two general classes of wall proteins are structural proteins and enzymes, and two major classes of structural proteins are the hydroxyproline-rich glycoproteins (HRGPs) and the glycine-rich proteins (GRPs). Included among the HRGPs are extensins (EXTs), proline-rich proteins (PRPs) and the arabinogalactan proteins (AGPs) (Kieliszewski et al., 2011; Showalter et al., 2010), and their expressions are regulated developmentally (Zimmermann et al., 2004). EXTs are among the first cell wall material detected in new cell walls, i.e., the cell plate (Hall and Cannon, 2002), and there are 20 classical EXTs in *Arabidopsis* (Saha et al., 2013). When EXTs are secreted into the cell wall, they become insolubilized through intra- and inter-molecular cross-links (Merkouropoulos et al., 2003). Merkouropoulos et al. (2003) also documented that Ext1 responds to various stress conditions in roots and inflorescences of Arabidopsis, such as wounding, pathogen infection, exogenously supplied salicylic acid, methyl jasmonate, auxins and brassinosteroids. Since stress conditions induce cross-linking of Ext1 (AT1G76930) and suppression of APY1 and APY2 induces stress-like changes in seedlings, I hypothesized that cross-linking of Ext1 in cell wall would be affected by apyrase suppression, and this would change its protein size. I tested this hypothesis using an Ext1 antibody to carry out Western Blots of IBP (ionically bound protein) and CBP (covalently bound protein) in cell walls of primary roots. The result confirms that Ext1 is insolubilized as a covalently bound protein in cell wall, and that its size is increased by apyrase

suppression (Appendix Figure 1). It seems likely that increased cross-links of Ext1 could be the cause of its bigger size, however, to clear what exactly makes the band shift, more study should be performed.

Although UDP-galactose transport in the Golgi is not changed by reduced apyrase expression, the galactose content of cell walls is significantly increased not only in R2-4A seedlings, but also in *apy1* and *apy2* mutant seedlings (Chiu et al., 2012). Many reports have addressed the cytotoxicity and deleterious physiological effects of free galactose, such as the growth inhibition of roots and shoots, and the inhibition of auxin biosynthesis and transport induced by a galactose-promoted increase in ethylene production (Egert et al., 2012; Rosti et al., 2007; Seifert et al. 2002; Sherson et al. 2000; Yamamoto et al., 1988). Despite all those findings, the exact mechanism of galactose toxicity in plants is still largely unknown. To evaluate the effect of galactose on root growth, I tested the relationship between extra galactose and lignification in primary roots. A significantly different pattern of lignification was observed in roots of WT and induced R2-4A mutants treated with 10mM D-galactose without sucrose (Appendix Figure.2), while the pattern was not changed in roots of WT and R2-4A treated with 10mM D-galactose with 1% sucrose (data not shown). This result rules out that the growth inhibition is caused by osmosis difference due to excess galactose in roots.

In addition to their essential roles in carbon and energy metabolism, sugars also play important roles in signal transduction as primary messengers (Rolland et al., 2002). For instance, sugar sensing and signaling mechanisms are related to mitotic activity (Borisjuk et al., 1998), and, correspondingly, the expression of D-type cyclin in *Arabidopsis* is regulated by sugars (Riou-Khamlichi et al., 2000). The lignin-staining results showed that treatment with 10 mM D-galactose without sucrose increases lignification of cells in the meristematic zone (Appendix Figure.2 B and

C). Taken together, our data suggest that a modified signal pathway caused by sugars such as galactose would be involved in lignification during the process of the growth inhibition induced by apyrase suppression.

As noted in chapter 2 (Fig. 2.6), all the growth and morphology changes induced by the suppression of APY1 and 2 cannot be mimicked by increasing the [eATP], or by apyrase inhibitors. Thus, even though suppressing APY1 and APY2 expression increases eATP levels and inhibits cell growth, it seems probable that the R2-4A root phenotype may not be due to sole action of an increase in the eATP concentration. Additional research would be needed to determine how many of the gene expression changes induced by the suppression of *APY1* and *APY2* in R2-4A seedlings are attributable to changes induced by the functions of these apyrases (e.g., Golgi functions) that are not related to signaling changes induced by increased [eATP].

A major breakthrough in the field of eATP signaling has been the recent identification of a receptor for eATP in Arabidopsis (Choi et al., 2014). This receptor is a lectin receptor kinase, LecRK-I.9, and was given the name DORN1 (Does not Respond to Nucleotides 1). It is needed for ATP-induced rapid changes in $[Ca^{2+}]_{cyt}$, downstream changes in mitogen-activated protein kinase, and gene expression changes related to wound responses. Relevant to data reported here, this lectin receptor kinase plays an important role in fungal pathogen resistance (Bouwmeester et al., 2014). Increased ligand concentrations often induce changes in the level or activity of its receptor (Chen et al., 2007). Although suppression of apyrase, which increases the [eATP], does not significantly alter the transcript level of DORN1, it will be valuable to learn whether apyrase suppression alters the activity, turnover or rate of endocytosis of DORN1.

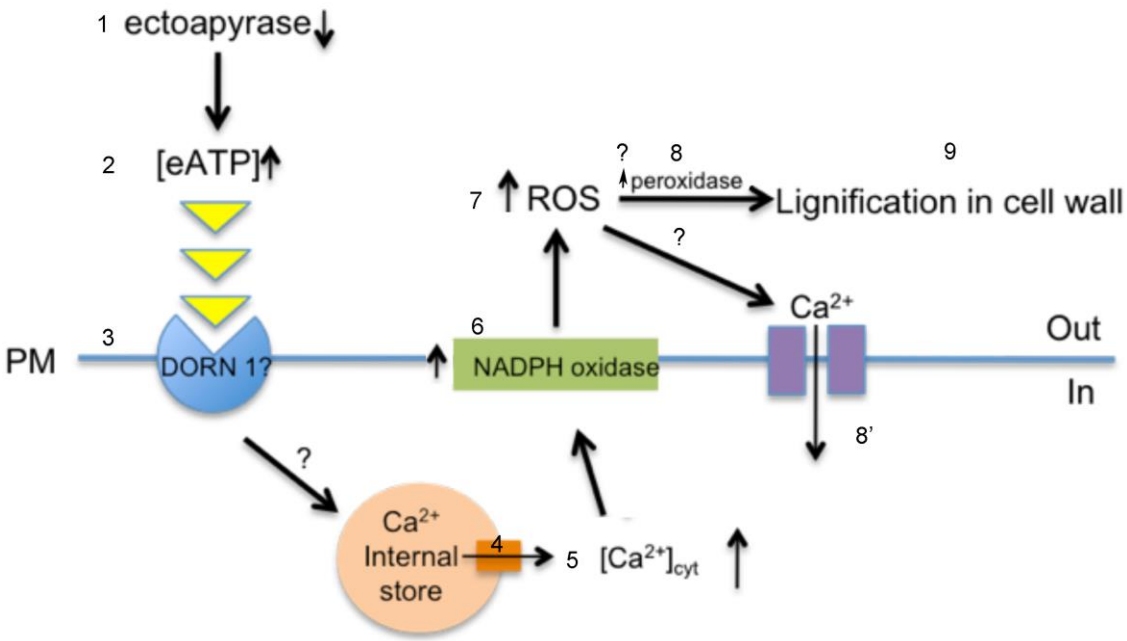
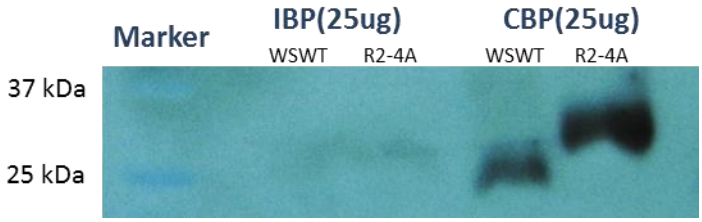


Figure 4.1. Hypothetical model of sequence of events that could connect the suppression of *APY1* and *APY2* to the increased lignification of cell walls, as reported in this dissertation. The events are postulated to begin with decreased ectoapyrase activity (1) and increased [eATP] (2). Major uncertain steps are whether DORN1 is the eATP receptor (3) for the downstream steps (4-9), and, if so, how its activation results in increased $[Ca^{2+}]_{cyt}$ (5). It is also not clear yet how increased ROS (7) activates gene expression changes, such as increased expression of wall peroxidase genes (8), and how ROS promotes increased uptake of Ca^{2+} into cells (8'), which appears to occur near the same time as ROS-induced gene expression changes.

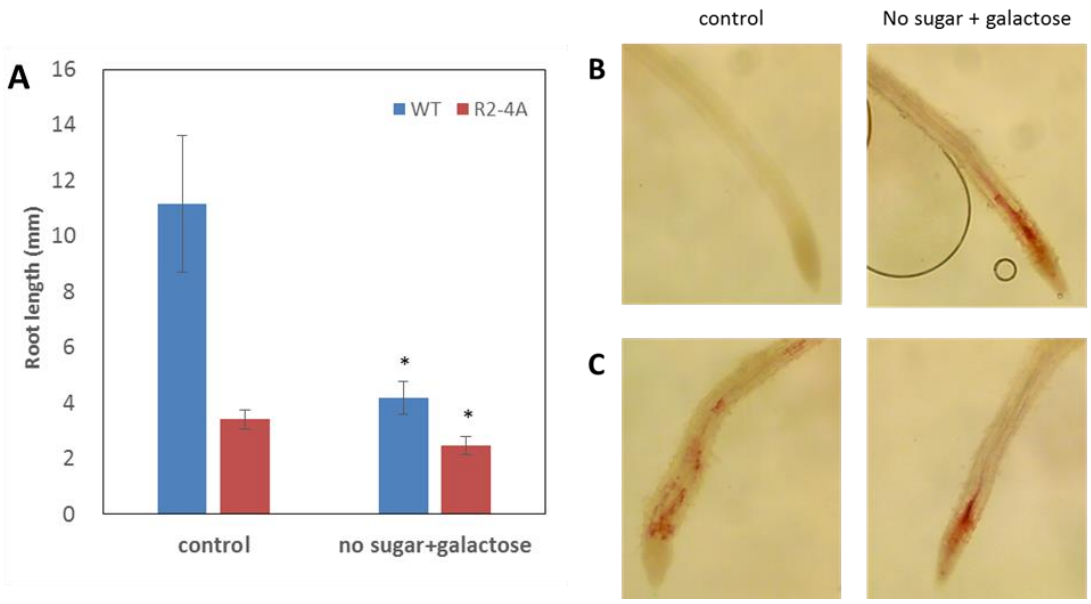
Appendix



Appendix Figure 1. Western blotting with Ext1 antibody. Same amount of IBP (ionically bound protein) and CBP (covalently bound protein) of WT and R2-4A wall proteins were loaded. Image is representative of two biological repeats.

Methods: Methods for extraction of wall proteins were as described in Chapter 3. Immunoblot methods were as Wu et al. (2007). Goat polyclonal IgG to Arabidopsis Extensin1 was used in accord with instructions from manufacturer, Santa Cruz (SC-15899)

Interpretation: Although this result is consistent with the interpretation that suppression of apyrase induces a shift in the molecular mass of extensin, it is not clear whether this is the result of increased cross-links of extensin with another protein. Direct assessment of iso-dityrosine cross links in CBP in induced R2-4A mutants compared to WSWT would help resolve this question.



Appendix Figure 2. The effect of galactose on primary roots of WT and R2-4A. (A) 6-d-old root length of WT and R2-4A with/without treatment. Control is 1% sugar and no additional D-galactose. 10 mM D-galactose treatment and/or no sugar treatment was performed to test root growth effect. (* p value $\leq$ 0.001 compared to each control, n $\geq$ 42). Lignin staining of primary roots WT (B) and R2-4A (C).

Methods for lignin staining and measurements of root length were as described in Chapter 3.

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